Modification of a whole-cell biosensor to detect toxins by SciTox™ rapid DTA assay.

A thesis

Submitted in partial fulfilment

of the requirement for Degree of

Doctor of Philosophy

at

Lincoln University

New Zealand

by

Song Wenfeng

Lincoln University

2012

Publications

Published:

1. **Title** - Comparison of three genetically modified *Escherichia coli* biosensor strains for amperometric tetracycline measurement.

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Journal – Biosensors & Bioelectronics 35 (1): 69-74

Date – 15 May 2012

My contribution to the manuscript included intellectual input into experimental design, all laboratory work, interpretation of the results and drafting the manuscript.

2. **Title** - Rapid Toxicity Assessment testing by using freeze-dried bacterial cells and electrochemical detection.

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Journal – Applid Microbiology and Biotechnology

Accepted date: 08/01/2013

My contribution to the manuscript included intellectual input into experimental design, all laboratory work, interpretation of the results and drafting the manuscript.

Manuscripts submitted:

1. **Title -** The improvement of genetically modified *Escherichia coli* biosensor for amperometric tetracycline measurement.

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Journal - Biosensors & Bioelectronics

Submission date: 28/08/2012

My contribution to the manuscript included intellectual input into experimental design, all laboratory work, interpretation of the results and drafting the manuscript.

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

Modification of a whole-cell biosensor to detect toxins using the SciTox™ rapid DTA assay

by Wenfeng Song

Pollution of soil and water caused by human activity such as agriculture and industry is an issue of great global concern. In order to mitigate, eliminate or avoid pollution it is necessary to be able to accurately measure and monitor it. Therefore, rapid and sensitive methods which can detect and quantify environmental toxicants are being developed.

The SciTox™ rapid mediated direct toxicity assessment (DTA) assay was developed by Lincoln Ventures Limited. It is a rapid catalytic microbial method in which the natural co-substrate, oxygen, is substituted by a synthetic co-substrate or mediator. Transfer of electrons from the mediator to an electrode poised at a suitable voltage can generate a measurable current and is used to quantify the magnitude of respiration inhibition and indirectly the toxicity.

The SciTox™ assay has two major constraints; the stability of whole cells and a lack of specificity to a target analyte. These two aspects are addressed in this thesis.

Part A: Three microorganisms, *Acinetobacter calcoaceticus* (*A. calcoaceticus*), *Escherichia coli* (*E. coli*) and *Pseudomonas putida* (*P. putida*) were evaluated as biocomponents in a whole-cell based bio-assay. Each of these bacteria was prepared in two forms, as freshly cultured cells and as freeze dried cells. In order to compare the storage efficiency of different cryoprotectants, each freeze-dried bacterial strain was prepared in two forms, pre-treated with either polyethylene glycol (PEG) or sucrose / Tween 80, and stored at two different temperatures, 4 ˚C and -20 ˚C. This was to validate that, after optimum freeze dried storage conditions, freeze-dried cells exhibited a similar response to the standard toxicants, 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorophenol (3,5-DCP) as their freshly cultured counterparts. In the freeze dried cell storage study, sucrose / Tween 80 was more efficient in maintaining the cell

viability. In contrast, PEG was more suitable for SciTox™ toxicity assay because it produced cells with more conventional EC_{50} values (closer to fresh ones). After one and two-month storage at either 4 ˚C or -20 ˚C, the viability of the cells decreased significantly but showed a similar dose-response curve, when tested in the DTA SciTox™ assay following a protocol identical to that applied to the fresh cell counterparts. After three-month stored at -20 ˚C, all three freeze-dried strains could be used to assay the toxicity of standard toxicants but at 4 ˚C, *A. calcoaceticus* and *E. coli* lost their activity in SciTox™ assay. Bacterium *P. putida* was the most resistant to freeze-drying and subsequent storage, and also the most stable strain in SciTox™ DTA assay compared with the other two. This is the first time the shelf-life of freeze-dried cells has been evaluated in a commercial toxicity assay.

Part B: The objective was to develop a biosensor strain of *E. coli* that is specific for detecting and quantifying antibiotics in milk samples by genetically engineering the bacterial component of the SciTox™ assay. The SciTox™ assay in its current configuration, measures toxicity nonspecifically and therefore cannot distinguish between toxicants. In order to create a bio-assay that can identify and quantify specific toxicants at concentrations lower than inhibitory levels, the SciTox™ assay was re-engineered by using specific promoters and reporter gene fusions for gene induction in response to a specific toxicant. Four separate strategies were investigated using four different reporter genes, *lacZ*, *selA*, *ompF* and *nuoA*. All strategies were based on gene induction in response to a specific toxicant. The *Tn10 tetA* promoter, de-repressed in response to the presence of tetracycline (Tet), and the *copA* promoter, activated in the response to the presence of copper (Cu) and silver (Ag), were used as the model systems. In each reporter system, the presence of Tet (*tetA* promoter regulated systems) or the presence of Cu and/or Ag (*copA* promoter regulated systems) led to increased expression of the reporter gene which produced an increase in respiratory activity and therefore an increased SciTox™ signal.

In conclusion, the three microorganisms, *A. calcoaceticus*, *E. coli* and *P. putida* could be freeze-dried and stored at -20 ˚C for up to 3 months and still be useful in the SciTox™ assay. Results of re-engineering the SciTox™ assay suggested that two genes (the *ompF* and the *nuoA*) could be used as reporter genes to improve the sensitivity and specificity of SciTox™ DTA toxicity assay. To our knowledge, this is

the first time *nuoA* gene, or any other respiratory gene, has been used as the reporter gene for an amperometric biosensor.

Key words: SciTox^{TM}, biosensors, freeze dry, potassium ferricyanide, 2,4-DCP, 3,5-DCP, *A. calcoaceticus*, *E. coli*, *P. putida*, *lacZ*, *selA*, *nuoA*, tetracycline, β-galactosidase, selenocysteine synthase, NADH dehydrogenase I

Acknowledgements:

I would like to thank my supervisors, Professor Ravi Gooneratne, Dr Neil Pasco and Dr Richard Weld. They gave a lot of help on both science fields and English. Thank you for helping me with my draft chapters.

Special thanks to Nick Glithero and Simon Lee, for their advice, technical assistance and support during the experiment at stages of this research.

Thank you to family and friends for their support. Finally, I like to thank Lincoln Ventures Ltd for their financial contribution and Lincoln University for the award of a PhD scholarship that allowed me to undertake this PhD degree.

Abbreviations

A. calcoaceticus	*Acinetobacter calcoaceticus*
Amp	Ampicillin
BSM	Basal Salt Media
2,4-DCP	2,4-dichlorophenol
3,5-DCP	3,5-dichlorophenol
DL	Detection limit
DMM	Davis Minimal Media
DTA	Direct Toxicity Assay
E. coli	*Escherichia coli*
ETC	Electron transport chain
GMO	Genetically modified organismes
Kan	Kanamycin
KFC (II)	Potassium hexacyanoferrate
KFC (III)	Potassium hexacyanoferrite
LB	Luria Broth
NB	Nutrient Broth
NOEL	No observed effect level
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
P. putida	*Pseudomonas putida*
Se	Selenium
Tet	Tetracycline

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CHAPTER 1: INTRODUCTION

As a result of human and technological development, a wide range of man-made chemicals and their by-products formed in industrial or combustion process have been, and still are, released into the environment. Consequently, the pollution of soil and water caused by agriculture, industry (including war industry), and /or by accidental contamination with fuels, oils, solvents, heavy metals and terrorist attacks etc, is becoming an issue of great global concern. Therefore, rapid and sensitive methods which can detect and quantify such toxicants are being developed (Kim et al. 2005).

Accurate measurement of environmental chemicals is important for environmental monitoring framework. Generally, low concentrations of chemicals can be detected by analytical methods, such as high performance chromatography (HPLC), liquid chromatography (LC), and heavy metals by atomic absorption spectroscopy. However, these methods are expensive and time consuming, and also have the disadvantage that they do not take into account the bioavailability of compounds (Thouand et al. 2003).

Environmental regulatory agencies are now calling for fast and cost-effective analytical techniques including biosensors to be used in environmental monitoring. A biosensor is defined as a device that detects, records, converts, processes, and transmits information regarding a physiological change or process, or a device that uses biological materials to monitor the presence of various chemicals in a substance (Rodriguez-Mozaz et al. 2005b). They can be viewed as an interface medium between three entities: living cells (such as bacterial cells), an artificial system (e.g. computer), and the environment.

Direct toxicity assessment (DTA) based on using the whole cell microbial biosensors offers a powerful approach to environmental monitoring (Tizzard et al. 2004). This assessment is not based on identifying the chemicals present, but measuring the impact of toxic chemicals on biological materials resident in the environment (Pasco et al. 2005). The reason for choosing bacteria instead of animals is because of ethical reasons. In addition, bacteria offer other advantages including low cost, great stability, broad spectrum of activity, long shelf life, high adaptability, and a capacity to be genetically modified easily and inexpensively (Rogers 2006).

The SciTox™ rapid mediated DTA assay was developed by Lincoln Ventures Limited (Pasco et al. 2001). It is a rapid catalytic microbial method in which the natural co-substrate, oxygen, is substituted by a synthetic co-substrate or mediator. The mediator is normally a low molecular weight redox active specie. They can replace the natural electron acceptors in the Electron Transport Chain (ETC) and bypass the oxygen reduction step (Pasco et al. 2005). Mediators can shuttle electrons from the bacterial ETC to an external electrode. For example, potassium hexacyanoferrate (KFCIII) is well known as a redox mediator that can couple with cellular respiration. The electrons released into the ETC through respiratory activity will be transferred to the mediator (e.g. KFCIII) via membrane bound dehydrogenase enzymes that span the plasma membrane. This process leads to the accumulation of reduced potassium hexacyanoferrite (KFCII) mediator in solution. Transfer of electrons from the reduced mediator to an electrode poised at a suitable voltage can generate a measurable current.

The basic bacterial toxicity assay principle is based on comparing the magnitude of a measurable current produced by microbial cells exposed to a toxicant at a fixed concentration relative to a control produced by control healthy microbial cells (Pasco et al. 2005).

In bacterial assays, the toxic effects are represented by using the index of the measurable attributes such as LC_{50}, EC_{50} or IC_{50}. LC_{50} represents the concentration of toxicant at which 50% of microorganisms die. EC_{50} is the effective concentration at which 50% of bacteria are affected in terms of mobility, growth inhibition etc (Pasco et al. 2005). IC_{50} is the inhibition concentration which decreases the respiration rate by 50%. It is well known that different species of micro-organisms show different effects even when they are exposed to the same toxicant.

Whole cell bioassays, such as the SciTox™ assay, are confronted by two major constraints:

1. the stability of whole cells, and
2. a lack of specificity to a target analyte.

Investigation of these two constraints forms the basis of my Ph D research which has hence been divided into two parts, A and B.

Part A: The stability of three bacteria, *A. calcoaceticus, E. coli* and *P. putida*, was evaluated as biocomponents in whole-cell based bioassay. Each of these bacteria was prepared as freshly cultured and as freeze dried cells. Each freeze dried bacterial strains was treated with cryo-protectants polyethyleneglycol (PEG) and sucrose with Tween 80. The freeze dried cells were sealed in bottles and placed in a desiccator, and stored at two different temperatures (4 and -20˚C, separately). These cell preparations were used to test the following:

(i) Whether freeze dried cells would have a similar response to two standard toxicants, 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorophenol (3,5-DCP), as compared to their freshly cultured counterparts;

(ii) To compare the efficacy of the two cryo-protectants and two storage tempeatures on the stability of the freeze dried cells in SciTox^{TM} toxicity assay when exposed to 2,4-DCP and 3,5-DCP.

The basic principle of SciTox^{TM} is that electrons from the respiratory activity of bacterial cells are transferred via the electron transport chain to the mediator KFC III. Re-oxidation of microbially reduced mediator (KFCII) generates a measurable current. By comparing the ratio of the re-oxidation current detected in the presence and absence of toxins, it is possible to gauge the index of respiratory inhibition, which is proportional to the toxin concentration.

Part B: To improve the specificity, a specific biosensor was constructed by genetically engineering the bacterial component (initially *E.coli*) used in the SciTox^{TM} assay. Typically, these whole-cell biosensors depend on expression of reporter genes from gene promoters that are inducible by target analytes. Analyte-specific whole-cell biosensors have been developed to detect a range of compounds and environmental stress including antimicrobial agents (Bianchi and Baneyx 1999), gamma irradiation (Min et al. 2000), genotoxins (Belkin et al. 1997) and oxidative stress (Belkin et al. 1996), etc.

Based on the current configuration, the SciTox^{TM} assay measures toxicity non-specifically and therefore cannot distinguish between toxicants. In order to create a bio-assay that can identify and quantify specific toxicants at concentrations lower than inhibitory levels, the SciTox^{TM} assay was re-engineered. Four separate strategies were devised to achieve this objective. They were based on gene induction in response to a

specific toxicant. The antibiotic tetracycline (Tet) and the *Tn10 tetA* promoter, which is de-repressed in response to the presence of Tet, was used as the model system.

In the first strategy, the quantity of metabolisable carbon available to the bacteria in the SciTox^{TM} assay was manipulated by a method used previously in yeast (Lehmann et al. 2000; Tag et al. 2007). In this method, lactose was used as the sole carbon source for bacteria in the SciTox^{TM} assay. *E. coli* strains lacking the *lacZ* gene do not produce β-galactosidase and are incapable of utilizing lactose as a carbon source. By re-introducing the *lacZ* gene fused to the *tetA* promoter into these cells, metabolisable carbon is only available to the cells in the presence of Tet.

In the second strategy, instead of inducing the expression of a specific respiration gene in the presence of the toxicant, the overall redox activity of the respiratory enzymes was manipulated. For this method, the *selA* gene, which encodes the enzyme selenocysteine synthase (Tormay et al. 1998a), was fused to the *tetA* promoter and transformed into a *selA* knock-out *E.coli* mutant. The *selA* gene catalyzes the reaction of serine to selenocysteine conversion on Ser-tRNA^{Sec} by using monoselenophosphate as the selenium (Se) donor (Allmang et al. 2009; Forchhammer et al. 1991). Selenium atoms have similar properties to sulphur (S) atoms. However, by substituting for S, the catalytic rate of seleno-proteins will be significantly increased (Tormay et al. 1998a).

In the third strategy, the passive diffusion efficiency of a mediator (KFCIII) across the out membrane of *E. coli* sensor cells was manipulated. For this method, *ompF* gene was fused to *tetA* promoter and transferred into the *ompF* knock-out *E. coli* mutant. Thus the SciTox^{TM} related activity might be increased due to the improved entry efficiency of KFCIII through cell membrane in the presence of Tet.

In the fourth strategy, a key respiration gene was placed under the control of the *tetA* promoter such that, in the presence of Tet, the gene was expressed and the cellular respiration rate increased. For this method, the *nuoA* gene, which encodes a protein that is directly involved in bacterial respiration was used. The *nuo* operon encodes the enzyme NADH dehydrogenase I, which catalyses the transfer of electrons from NADH into the start of the respiratory chain (Wackwitz et al. 1999), and functions in both anaerobic and aerobic conditions (Calhoun et al. 1993).

While *lacZ* has previously been used as a reporter gene in an amperometric bioassay (Tag et al. 2007), *selA*, *ompF* and *nuoA* are novel reporter genes. To our knowledge, this is the first report in which a respiratory gene was used as a reporter gene in an amperometric biosensor. The results indicate that this approach can produce a highly sensitive detection system.

a) Aims of this study

- **Part A**: To investigate the stability of freeze dried bacteria as biocomponents in whole-cell based SciTox^{TM} mediated DTA bioassay.

- **Part B**: To engineer toxicant specificity into the SciTox^{TM} rapid DTA assay by using inducible respiratory reporter genes.

b) Hypotheses

Part A:

- The freeze dried cells exhibit similar SciTox^{TM} response to the standard toxicants (2,4-DCP/3,5-DCP) as the cultured fresh cells for up to three months.

- The freeze-dried cells treated with sucrose Tween 80 are more stable and exhibit similar SciTox^{TM} response to the standard toxicants (2,4-DCP/3,5-DCP), compared with the freeze-dried cells with PEG for up to three months.

- The freeze-dried cells stored at -20 ˚C exhibit a greater shelf life than 4 ˚C and therefore exhibit a similar SciTox^{TM} response to the standard toxicants (2,4-DCP/3,5-DCP) as the cultured fresh cells for up to three months.

Part B:

- That respiration reporter gene (such as *ndh, nuoA*) induction in *E. coli* can be used to measurably increase or decrease bacterial respiration as measured by the SciTox^{TM} assay.

- That the SciTox^{TM} assay can be modified to specifically detect sub-inhibitory levels of antibiotics (e.g. tetracycline).

- That the induced SciTox^{TM} response is proportional to the antibiotic concentration (dose-response quantification).

c) Objectives

Part A:

- To test the shelf stability of freeze dried microorganisms, *A. calcoaceticus*, *E. coli* and *P. putida* treated with two different cryoprotectants (PEG and sucrose with Tween 80) and at two different storage conditions (4 ˚C and -20 ˚C) over three different time periods (one, two and three months) and measure the respiritory response using SciTox^{TM} to the standard toxicants, 2,4-DCP and 3,5-DCP.

- To compare the SciTox^{TM} response of the freeze-dried *A. calcoaceticus*, E. *coli* and *P. putida* treated with two different cryoprotectants (PEG and sucrose with Tween 80) and at with two different storage conditions (4˚C and -20 ˚C), at three different time periods (one, two and three months), with cultured fresh cells in SciTox^{TM} rapid DTA assay.

Part B:

- To find and test promoter elements that are regulated (up or down) by the antibiotic concentrations in water samples and are suitable for use in biosensors.

- To find and test genes which regulate *E. coli* respiration and thereby change the SciTox response.

- To construct and test a biosensor strain of *E. coli* for detecting and quantifying antibiotics in milk samples

CHAPTER 2: LITERATURE REVIEW

2.1 Biosensors

A wide range of man-made chemicals and their by-products formed in industrial or combustion process have been, and are being, released into the environment. Monitoring these contaminants in the air, water and soil is an important component in understanding and managing risks to human health and the environment. Accurate measurement of the concentrations and bioavailability of environmental pollutants constitutes a major tool for environment monitoring. Generally, low concentrations of chemicals can be detected by very sensitive methods, such as high performance liquid chromatography (HPLC), liquid chromatography (LC), etc. However, these methods are expensive and time consuming, and also have the disadvantage that they do not take into account the bioavailability of compounds (Thouand et al. 2003). Biosensors and bioanalytical methods developed for environmental monitoring offer some advantages over conventional techniques including their ability to measure bioavailability and their potentially inexpensive manufacture (Rogers 2006).

A biosensor is an analytical device, which can convert biological recognition of a chemical into a detectable signal (Gronow 1984), thereby allowing indirect quantification of the chemical. Normally, biosensors contain three major components (Figure. 2.1):

- Sensitive biological recognition element such as tissues, organelles, enzymes, microorganisms.
- Transducer/ detector element that converts a biochemical reaction into a signal.
- Associated electronics/ signal processors that display the signal.

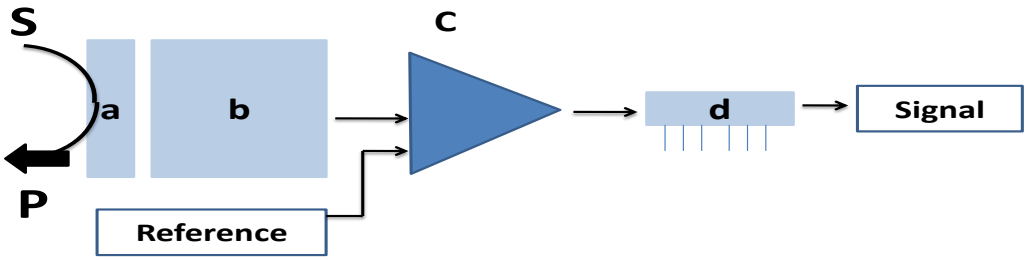

Figure 2.1. Basic principle of Biosensors; a: biocatalyst [converts the substrate (S) to product (P)]; b: transducer determines the reaction and converts it into an electrical signal; c: the transducer signal is amplified; d: amplified signal is processed; e: signal display.

Recently, biosensors have been widely used in agriculture monitoring (Velasco-Garcia and Mottram 2003), groundwater screening (Rodriguez-Mozaz et al. 2005a), ocean (Kroger and Law 2005) and global environmental monitoring (Rodriguez-Mozaz et al. 2005b). There are many different types of biosensors, such as enzyme-based biosensors, antibody-based biosensors, and whole-cell based biosensors.

2.1.1 Enzyme-based biosensor

An enzyme-based biosensor is an analytical device which combines an enzyme with a transducer to produce a signal proportional to a specific analyte concentration (Mulchandani 1998). Based on their substrate-specificity and catalytic activities, enzymes have been used as sensing elements in biosensors (Verma and Singh 2005). The enzyme-based biosensors have been employed for detection of a wide range of environmental compounds, such as insecticides, phenol compounds and heavy metals. For example, the genetically modified acetylcholinesterase (AChE) from *Drosophila melanogaster* could detect the insecticide omethoate with a limit of detection down to 10^{-11} µM (Bucur et al. 2005) due to the inhibitory activity of the insecticide. A

recently developed amperometric cytochrome c_3- based biosensor could detect heavy metal Cr (VI) with detection limit of 0.2 mg L^{-1}, due to a measurable current generated through reduction of Cr (VI) to Cr (III) catalyzed by the cytochrome c_3 enzyme (Michel et al. 2006). Tyrosinase, is an enzyme that catalyses the oxidation of phenol compounds (e.g. tyrosine). Based on this reaction, a tyrosinase-based biosensor was developed to measure phenols in environmental samples (e.g. bisphenol A) at concentrations in the low μM range (Andreescu and Sadik 2004).

The enzyme-based biosensors offer several advantages, including the ability to modify the catalytic properties or substrate specificity by genetic engineering, and catalytic amplification of the biosensor response by modulation of the enzyme activity with respect to the target analyte (Rogers 2006). However, there are limitations such as limited interaction between environmental compounds and enzymes and lack of specificity in differentiating among compounds of similar class (Rogers 2006).

2.1.2 Antibody-based biosensors

An antibody-based biosensor (immunosensor) is an analytical device that uses an antibody to interface to a signal transducer to measure a binding event. The antibodies used in a biosensor can specifically bind to individual compounds or groups of structurally related compounds with a highly affinity (Rogers 2006). Antibody-based biosensors have been used for the detection and quantification of environmental analytes, such as biotoxins and heavy metals. For example, in a microchip format system, the antibodies immobilized on a glass chip using hydrogel-based chemistry could detect six biotoxins (ricin, viscumin, staphylococcal enterotoxin B, tetanus toxin, diphtheria toxin and anthrax toxin) at a level of ng ml^{-1} (Rubina et al. 2005). In another study, an inhibition immunoassay has been used for the analysis of cadmium (Cd) using anti-cadmium monoclonal antibodies which bind tightly to Cd-ethylenediaminetetraacetic acid (EDTA) complex. The assay could detect Cd with a sensing range of 0.06 – 4.45 μM (Khosraviani et al. 1998).

Antibody-based biosensors offer several advantages over traditional methods, such as high sensitivity, selectivity and species-specificity (Verma and Singh 2005). However,

there are still several limitations, such as the complexity of assay formats and limited compounds detected in any individual assay (Rogers 2006).

2.1.3 Whole-cell based biosensor

A whole-cell based biosensor is a device that uses either prokaryotic or eukaryotic cells as biocomponents linked with a transducer to produce a signal that responds to changes in cellular metabolic processes (e.g. respiration and fermentation) in response to external physical or chemical conditions. The whole-cell based biosensors are widely used for environmental monitoring (assay toxicity of environmental analytes). Whole-cell based biosensors can be roughly divided into two main types on the basis of their specificity to the analytes. They are either substrate-specific or non-specific.

2.1.3.1 Non-specific whole-cell biosensors

Whole cells can be used to identify and respond to both physical and environmental factors due to their subcellular metabolism mechanisms (Pasco et al. 2011). Respiration is a fundamental cellular process that adapt and respond to physical and chemical changes, making it an ideal analytical signal. Many commercial toxicity assays (e.g. SciTox^{TM}, Microtox$^{®}$ and ToxAlert$^{®}$) use respiration to determine toxicity or to monitor other metabolic events (dos Santos et al. 2002; Hansen and Sorensen 2001; Tizzard et al. 2004). The energy transducing membranes of microorganisms, such as the plasma membrane of prokaryotes or the inner membrane of eukaryote mitochondria (Voet et al. 2006) have two distinct protein assemblies: the ATP synthase at which adenosine diphosphate (ADP) is converted to adenosine triphosphate (ATP) and the energy source for ATP synthesis, the ETC (Black 2008). In the ETC, energy from oxidation of carbon compounds such as glucose is used to pump protons across the plasma membrane. Electrons passing through the ETC are, in many cases, ultimately transferred out of the ETC to oxygen. Respiratory activity is generally reduced when bacterial cells are exposed to toxic substances, such as 3,5-DCP (Tizzard et al. 2004). The dose-response relationship of the respiration of bacterial strains to the chemicals can be measured by the change in the number of

electrons passing down the ETC. Some commercial toxicity tests, such as Microtox® (Hansen and Sorensen 2001) and ToxAlert® (dos Santos et al. 2002), have used transgenic bacteria containing an expressed *lux CDABE* operon from *Vibrio fischeri* to detect the presence of chemicals. Light emission produced by the organism is directly correlated to energy-production and respiration rate as the light-generating reactions require high energy use. The toxic compound or environmental sample inhibits bacterial respiration resulting in decreased production of light (Nivens et al. 2003). Such toxicity assays are non-specific, because any condition which decreases the metabolic activity of the biosensor bacteria, will decrease its respiration (Hansen and Sorensen 2001). This type of biosensor is termed as non-specific biosensor which is sensitive to a wide range of substances including metals, and xenobiotics (Beaton et al. 1999; Chaudri et al. 1999).

2.1.3.2 Specific whole-cell biosensors

The specific biosensors are developed and used to detect and quantify specific environmental substrates. These specific biosensors are achieved by exploiting natural endogenous pathways, such as specific chemical degradation pathways, and by creating new signalling pathways by genetic recombination or by modification of existing pathways (Pasco et al. 2011).

2.1.3.2.1 Metabolic stimulation by specific contaminants

Contaminants may be highly toxic to some organisms while simultaneously being innocuous or even nutritious to others organisms. Microorganisms that can metabolize such toxic compounds could be used as biocomponents in whole cell biosensors to detect specific toxicants through detection of stimulation of metabolism (Pasco et al. 2011). *Pseudomonas mendocina* has been used as a specific biocomponent to detect petroleum compounds (benzene, toluene, ethylbenzene and xylene [BTEX]), due to increased catabolism of the BTEX chemicals measured by the rate of resazurin dye reduction to a fluorescent product (resorufin) (Tizzard et al. 2006).

2.1.3.2.2 Detection of toxicant degradation products

Some microorganisms could degrade chemical compounds to an easily detected product (e.g. electroactive substance), due to their natural metabolic pathways (Pasco et al. 2011). These microorganisms can be potentially used as whole cell biosensors to detect specific toxicants. *Moraxella* sp. can degrade *p*-nitrophenol to hydroquinone. Therefore, *Moraxella* sp. has been used to specifically detect and quantify *p*-nitrophenol in conjugation with a carbon paste electrode, correlating the electrochemical oxidation current of hydroquinone formed in biodegradation of p-nitrophenol (Mulchandani et al. 2005).

2.1.3.2.3 Genetically engineered whole cell biosensors

Sensitive, substrate-specific biosensors can be developed using DNA technologies and genetic manipulation (Pasco et al. 2011). This type of biosensor is genetically engineered by fusing an inducible promoter to a reporter gene. Therefore, the expression of the reporter gene is regulated by specific substrate concentrations (Figure 2.2). In the absence of the specific analyte (inducer), repressor proteins bind to operator sequences in or near to the promoter and block RNA polymerase transcription from the promoter; an inducer interacts with the repressor protein, releasing the repressor from the operator and the reporter gene is subsequently transcribed from the promoter. Signal transduction produced by bioreporters mainly involves light detection of fluorescence or luminescence, colorimetric detection and amperometric detection (Pasco et al. 2011).

Commonly used reporter genes include *lacZ* (encodes β-galactosidase), *gfp* (encodes green fluorescent protein), *lucF* (encodes luciferase in eukaryote), and *luxCDABE* (encodes luciferase in bacterial cell). Among these, use of the entire *luxCDABE* gene cassette has been extensive because such a reporter gene does not require the addition of an exogenous substrate for signal production (Li et al. 2008). The genetically engineered whole-cell based bioluminescent bioreporters have been used to specifically detect and quantify a wide range of compounds, including heavy metals (e.g. lead), organic compounds (e.g. toluene) and antibiotics (e.g. tetracycline) (Hansen and Sorensen 2000; Li et al. 2008; Nivens et al. 2003). Bioluminescence

reporters exhibit a fast response time and high short-term sensitivities due to the catalytic nature of the bioluminescent protein (Li et al. 2008). In contrast, fluorescent reporters are very stable (Li et al. 2008) but less sensitive (Pasco et al. 2011). However, the applications of new techniques (e.g. laser-induced-fluorescence confocal spectroscopy) could improve the detection limits to a level similar to or even better than those obtained by bioluminescence reporters (Kohlmeier et al. 2007). The *lacZ* based colourimetric bioreporter is widely used in the blue-white assay that offers the advantage of simple visible inspection (Freire-Picos and Lamas-Maceiras 2006).

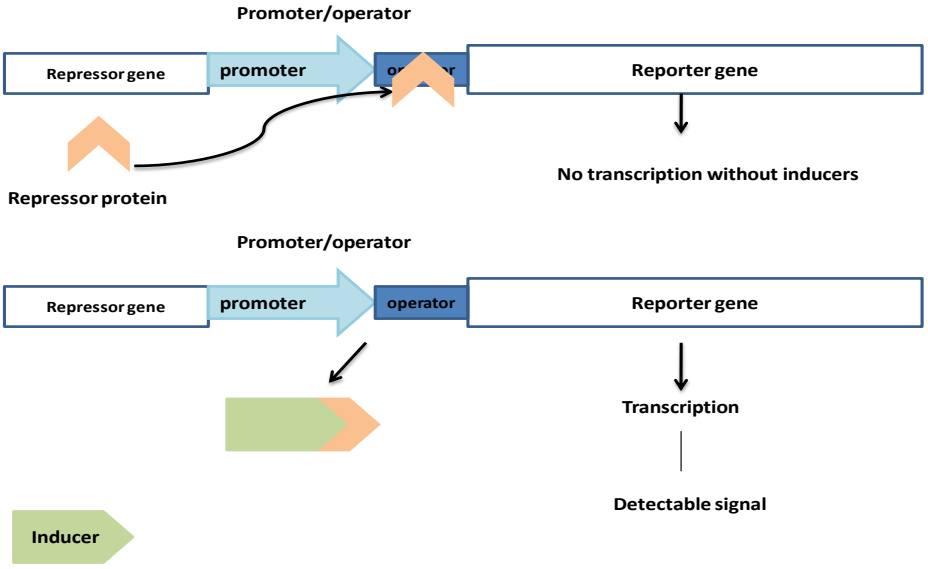

Figure 2.2. Example of an induction of a repressor type-specific biosensor. The inducer interacts with the repressor, releasing the repressor protein from operator of promoter and allowing transcription of reporter gene.

2.2 Inducible-promoter elements

2.2.1 *lac* operon

The *lac* operon is included and consists of three structural genes *lacZ*, *lacY* and *lacA*, and one repressor gene, *lacI*. The *lac* operon is an inducible operon. In the absence of the inducer, the repressor protein binds to the operator, blocking structural gene expression (Lewin 2008). When the repressor binds to the operator, it enhances RNA

polymerase affinity binding to the promoter by two orders of magnitude (Lewin 2008). However, the repressor causes the RNA polymerase to be stored at the promoter. RNA polymerase, repressor and DNA bind together and form the complex, which is blocked at the "closed" stage (Lewin 2008). The closed complex can become open by the removal of the repressor due to the addition of inducer, and which thereby initiates transcription (Ogorman et al. 1980).

The *lac* operon contains three operators, *O1*, *O2* and *O3*. *O1* is located just at the *lacZ* gene, the other two operators are located on either sides of *O1*; *O2* is 401 base pairs (bp) downstream of *O1* and *O3* is 92 bp upstream of *O1* (Narang 2007). *O1* is the main operator that has the strongest affinity for the repressor, whereas *O2* and *O3* can be termed "auxiliary" operators (Narang 2007). The repression is higher in the presence of the auxiliary operators which interact with the main operator, *O1*. LacI, the repressor protein, binds to *O1* and to one of the other operators, it will cause the DNA between the operators to form a rather short loop (Oehler et al. 1990). The loop structure can stabilize the binding of the repressor to *O1* (in the absence of inducer). For example, even if the repressor is detached from *O1* due to thermal fluctuations, the weak interaction of the repressor to *O2* or *O3* keeps it within a small neighbourhood of *O1*, thus increasing the probability that it rebinds to *O1* (Narang 2007).

In the *lac* operon, transcription of structural genes (*lacZYA*) is controlled by the repressor LacI, the product of the *lacI* gene. *lacI* has an independent transcription unit with its own promoter and terminator (Lewin 2008). In the presence of inducer (IPTG/lactose), the binding to the inducer leads to the allosteric change in lacI repressor, resulting in release of the repressor from the operators of the *lac* promoter (Narang 2007). Transcription is activated as soon as an inducer is added.

2.2.2 *Tn10* operon

The *Tn10* encoded tetracycline (Tet) resistant determinant mainly consists of the Tet resistance gene *tetA* and the repressor gene *tetR* (Beck et al. 1982; Gatz and Quail 1988; Postle et al. 1984). *TetA* encodes an inner membrane transport protein TetA, which facilitates the active efflux of Tet from the cytosol (Sambrook and Russell 2001). In the *Tn10* operon, the transcription of both *tetR* and *tetA* is controlled by a

shared regulatory region consisting of oppositely oriented, overlapping promoters P_A (promoter for *tetA*) and P_Rs (promoters for *tetR*) and two tandem operators *O1* and *O2* (Oehmichen et al. 1984). Repressor protein (TetR) encoded by *tetR*, can recognize both operators. The affinity of TetR with the two operators differs by a factor of 2 (Meier et al. 1988). There are two promoters P_{R1} and P_{R2} involved in P_Rs. The *tetR* gene can be transcribed by either P_Rs (P_{R1} and P_{R2}) (Meier et al. 1988). The *O1* overlaps with the -10 region of P_{R2} and is located in the region transcribed from P_{R1} while the *O2* overlaps with -35 consensus sequence of P_{R1} (Meier et al. 1988). Therefore, TetR binds to *O1* could reduce the transcription of tetR whereas occupation of *O2* with TetR may only affect the transcription from P_{R1} (Meier et al. 1988).

The *tetA* gene is more strictly regulated than the *tetR* gene by both operators (Meier et al. 1988). Expression from *tetA* could be repressed from P_A due to TetR binding to either *O1* or *O2*. The P_Rs are completely repressed when TetR binds to *O1*, whereas only slightly affected when TetR bind to *O2* (the operator that binds with highest affinity to the repressor). This indicates that both P_{R1} and P_{R2} will be repressed, when *O1* is occupied by Tet repressor. However, TetR binding to *O2*, may only repress the transcription from P_{R1} (Meier et al. 1988). The different regulatory properties of the two operators imply that *tetA* is more strictly regulated than *tetR*. As the level of repressor protein falls, through natural attrition and cell growth, the *tetR* gene becomes de-pressed and more repressor protein is produced. This system ensures that TetR is maintained at optimum levels in the cell and that TetA protein is only produced when specifically induced by Tet. This genetic switch is an excellent example of elegant, parsimonious biological regulation.

In the presence of Tet, TetR binds to Tet-Mg^{2+} complex, inducing conformational changes that reduce the binding constant of TetR to the operators by 6-10 orders of magnitude (Aleksandrov et al. 2008). The expression of both *tetA* and *tetR* therefore can proceed (Aleksandrov et al. 2008; Virolainen et al. 2008).

2.2.3 *CopA* promoter regulation

The *copA* gene encodes a copper-translocating P-type ATPase (CopA protein) (Rensing et al. 2000) driven by the copper (Cu)-inducible *copA* promoter (Stoyanov and Brown 2003). The CopA protein plays an important and unique role in Cu-resistant mechanism in *E. coli* (Stoyanov and Brown 2003). The protein CueR which is a MerR like regulator (a mercury [Hg] resistant regulator in a number of gram-negative and positive bacteria) (Hobman and Brown 1997) acts as the sole regulator of the expression of both *copA* and *cueR* in *E. coli* (Stoyanov et al. 2001). The CueR could interact with DNA (the operator) and RNA polymerase and form a ternary complex, which is then activated by binding of a single ion, Cu/Ag (Stoyanov et al. 2001). The CueR protein has a similar sequence to MerR and CueR containing a C-terminal metal-binding region which is also conserved in MerR (Outten et al. 2000). However, CueR lacks a cysteine (Cys) residue which is conserved at position 80 in MerR sequence. Additionally, the space between two other highly conserved Cys residues is shorter in CueR than in MerR (Outten et al. 2000). These differences in the metal binding domain of CueR might play roles in distinguishing between Cu/Ag and other metals (e.g. Hg) (Outten et al. 2000). However, gold (Au) has been reported as a mimic of Cu that could be recognized by the CueR metal binding site, and leading to activation of the *copA* promoter (Stoyanov and Brown 2003).

2.3 Amperometric biosensor

There are several different types of transducers used in whole-cell biosensors, including electrochemical, optical, photometric, calorimetric, acoustic and electronic. Electrochemical transducers which measure both generation and consumption of electrons during biochemical reactions are commonly used (Chaubey and Malhotra 2002). Electrochemical transducers can be further divided into amperometric, coulometric, potentiometric and conductometric biosensors (Lei et al. 2006). The SciTox^{TM} direct rapid DTA assay is an amperometric biosensor based system.

The amperometric biosensors measure the changes in the current at a working electrode due to direct oxidation of the products of a biochemical reaction. Ferrocene and its derivatives are mostly used as mediators in an amperometric biosensor

(Karyakin et al. 1994; Pasco et al. 2008). There are three electrodes involved in amperometric transducers, an auxiliary electrode, a working electrode and a reference electrode. The working electrode is used to measure the quantity of reduced mediator produced during the microbial incubation and it is poised at a fixed potential relative to a reference electrode. When a microelectrode is used as the working electrode, the limiting current will be proportional to the concentration of reduced mediator and the anodic current obtained 10 s after imposition of the applied potential was taken as the limiting current value.

2.3.1 Genetically modified whole-cell based amperometric biosensor

The blue-colour visual *lacZ* has been used as reporter gene and driven by a Cu-inducible *CUPI* promoter in yeast. In the presence of Cu, the *CUPI* promoter was induced, therefore the *lacZ* gene was expressed (Lehmann et al. 2000). The *lacZ* gene encodes the enzyme β-galactosidase, which can cleave lactose to galactose and glucose. In cells that cannot catabolise lactose, which are incubated with lactose as the sole carbon source, expression of *lacZ* leads to the availability of simple sugars which can be metabolized as a carbon source. Catabolism of these sugars, increases respiration in turn causing a decrease in oxygen concentration of the medium. The decrease in oxygen can then be measured with an oxygen electrode (Lehmann et al. 2000; Tag et al. 2007). This assay could specifically detect Cu^{2+} at a detection sensing range of 0.5 – 2.5 mM. The assay has been further studied in conjunction with the flow injection analysis (FIA), and the sensing detection range of Cu^{2+} was as low as 1.5 -7 mg L^{-1} (23.4 – 109.4 μM) (Tag et al. 2007).

2.3.2 Electron Pathway of Bacterial cells

Electron transfer from bacteria to external electron acceptors via the ETC is a biologically important phenomenon that is increasingly being harnessed as a measure of cellular activity in assays, such as the SciTox™ assay. The ETC plays a major role in respiratory activity of all living cells. In eukaryote cells, the most common electron donor is NADH, and the natural terminal electron acceptor is oxygen (Voet et al.

2006). The high energy molecule ATP is synthesized from ADP catalysed by ATP synthase, driven by the energy from electrons being transferred down the ETC (Black 2008). In the ETC, energy from oxidation of carbon compounds such as glucose is used to pump protons across the membranes. In eukaryotes, the ETC is located on the inner membrane of the mitochondria. NADH (nicotinamide adenine dinucleotide, reduced form) and $FADH_2$ (flavin adenine dinucleotide, reduced form) act as electron carriers. The Complex I (NADH-CoQ oxidoreductase) accepts electrons from NADH, whereas the Complex II (succinate-CoQ oxidoreductase) accepts electrons from $FADH_2$. Electrons carried from complexes I and II to Complex III (CoQ-cytochrome *c* oxidoreductase) by coenzyme Q (CoQ / ubiquinone), and from Complex III to Complex IV (cytochrome *c* oxidase) by the peripheral membrane protein cytochrome *c*. In Complex IV, molecular oxygen is reduced to water by electrons and hydrogen ions (H^+).

In bacterial cells, the components of ETC can be found in the plasma membrane. Electron transfer along the ETC is used to pump protons out from the cytosol through protein complexes in the plasma membrane, but can flow back to the cell through the enzyme ATP synthase (Voet et al. 2006). In bacterial cells, the electron pathway is much more complicated than in eukaryote cells. Bacterial cells can use multiple ETCs. For example, the ETC of *E. coli* consists of 15 primary dehydrogenases and 10 terminal reductases (Unden and Bongaerts 1997). Since dehydrogenases and terminal reductases use quinone as a common substrate or redox mediator, each of the dehydrogenases could react with any of the terminal reductases (Unden and Bongaerts 1997). This offers many advantages including more opportunities for proton translocation across bacterial cell membrane, greater ATP yield per electron, adjusting oxidative phosphorylation to the availability of different energy sources and balancing ATP synthesis (Voet et al. 2006). In the ETC, bacterial cells can use different electron donors, different dehydrogenases, different oxidases, and several types of electron acceptors (Fenchel et al. 2006). Electrons can enter ETC at three different levels, dehydrogenase level, quinone level and as a mobile cytochrome electron carrier (Fenchel et al. 2006). In respiration, the natural terminal electron accepters are oxygen or oxidised compounds, such as NO (Ducluzeau et al. 2009).

Indeed, *E. coli* possesses a number of regulation systems for rapid response to availability of oxygen and the presence of other electron acceptors (Unden et al. 1995).

The adaptive response is coordinated by a group of global regulators, such as the ArcAB system (Shalel-Levanon et al. 2005). The ArcAB system is a two-component regulatory system that contains ArcA (the cytosolic response regulator) and ArcB (the transmembrane histidine kinase sensor) (Shalel-Levanon et al. 2005). The ArcB protein is activated when the microorganisms' growth conditions change from aerobic to anaerobic. The increased level of phosphorylated ArcA caused by activated ArcB induces the expression of enzymes involved in fermentation (Lin and Iuchi 1991; Unden et al. 1995), while repressing the expression of genes involved in ETC (Shalel-Levanon et al. 2005). Our previous study also demonstrated that a significant increasing of cell's respiratory activity was obtained from the *ArcA* knock-out *E. coli* strain (Weld et al. 2010).

2.3.3 Potential respiration reporter genes for SciTox™

2.3.3.1 *nuoA-N*

The *nuo* operon contains 13 genes, *nuoA-N*, which collectively encode for NADH dehydrogenase I, plays an important role in the *E. coli* respiration chain (Zhang et al. 2004). NADH dehydrogenase I in *E. coli* catalyzes the electron transfer reaction from NADH to ubiquinone (the first step in *E.coli* ETC) (Calhoun et al. 1993). *E. coli* contains an additional NADH dehydrogenase II, which is encoded by the *ndh* gene (Wackwitz et al. 2000). NADH dehydrogenase II catalyzes the same reaction as NADH dehydrogenase I, but does not couple the reaction to proton translocation (Calhoun et al. 1993). These two enzymes are synthesized and used under different conditions (Calhoun et al. 1993). NADH dehydrogenase II operates in aerobic respiration and is repressed under anaerobic conditions (Green and Guest 1994). In contrast, NADH dehydrogenase I functions in both anaerobic and aerobic conditions. The expression of *nuoA-N* is strongly growth-phase dependent (Wackwitz et al. 2000). The activity of NADH dehydrogenase I is maximal in the presence of fumarate and intermediate in aerobic conditions and minimal in fermentative conditions (Unden et al. 2002).

2.3.3.2 *sel* operon

The *sel* operon contains four structural genes, *selA, selB, selC* and *selD* (Sculaccio et al. 2008). The *sel* operon encodes for selenocysteine which is structurally similar to cysteine, but contains Se instead of S (Tormay et al. 1998a). However, selenocysteine is chemically more reactive than cysteine (Atkinson et al. 2011). Synthesis of selenoproteins in *E. coli* mainly consists of four steps. Firstly, *selC* encodes a specific selenocysteine tRNA, which is aminoacylated with L-serine by catalysis of seryl tRNA synthetase (Sculaccio et al. 2008). *SelD* encodes enzyme selenophosphate synthetase, which catalyzes synthesis of monoselenophosphate from selenide and ATP (Itoh et al. 2009). The *SelA* gene encodes selenocysteine synthase, which catalyzes the conversion of seryl-$tRNA^{Sec}$ to selenocysteinyl-$tRNA^{Sec}$ by using monoselenophosphate as the Se donor (Stock et al. 2010). The selenocysteine-specific elongation factor SelB, encoded by *selB* gene, forms a complex with selenocysteinyl-$tRNA^{Sec}$ and GTP (Atkinson et al. 2011). The complex is then transferred to the ribosome and inserted into a polypeptide at the position corresponding to a dedicated UGA codon on a selenoprotein-encoding mRNA (Stock et al. 2010).

2.3.3.3 *ompF* gene

E. coli contains a number of outer membrane proteins which may play roles in both specific and non-specific membrane transport of hydrophilic molecules (e.g. ions, amino acids and sugars.etc) (Nikaido and Vaara 1985). The outer membrane protein F (OmpF) encoded by the *ompF* gene, forms a large porin channel involved in passive diffusion across the outer membrane in Gram-negative bacteria, including *E. coli* (Bekhit et al. 2011; Garcia-Gimenez et al. 2011; Housden et al. 2010). The entry of antibiotics into bacterial cells is mainly regulated by the function of OmpF protein (Housden et al. 2010; Sambrook and Russell 2001). Therefore, up-regulation of ompF expression might enhance antibiotics (e.g. Tetracycline) and SciTox^{TM} mediator (e.g. KFCIII) entry efficiency through the cell membrane. The expression of *ompF* is regulated by the EnvZ-OmpR (Response regulator protein for osmoregulation which is encoded by the *ompR* gene) regulatory system (Chagneau et al. 2001). EnvZ is a transmembrane protein that could sense different environmental signals. Upon high

osmolarity, EnvZ phosphorylates itself at a histidine residue by using ATP (Igo and Silhavy 1988; Roberts et al. 1994), and then transfers the phosphate to aspartate of OmpR (Delgado et al. 1993). However, the mechanism by which EnvZ senses environmental change is still unclear. The phosphorylation triggers the conformational changes of OmpR (OmpR-P) (Kenney et al. 1995), but how this change affects OmpR control of *ompF* is not clear. However, OmpR could increase DNA binding affinity to *ompF* promoter due to its conformation reshaping, and enable interaction of OmpR with the α subunit of RNA polymerase and initiating transcription (Kenney et al. 1995).

2.3.4 SciTox™ rapid toxicity assay

The SciTox™ toxicity assay is a commercially available whole cell microbial assay which is developed for environmental monitoring. In the environmental monitoring area, the SciTox™ assays are mainly targeting biochemical oxygen demand (BOD) (Catterall et al. 2001; Catterall et al. 2003; Pasco 2005; Tizzard et al. 2004), and the rapid DTA assay (Pasco et al. 2008).

SciTox™ DTA assay is a rapid catalytic microbial method in which the natural co-substrate; oxygen is substituted by a synthetic co-substrate or mediator. The mediator is normally low molecular weight redox active species. They are capable of replacing the natural electron acceptors in the Electron Transport Chain (ETC) and thereby bypassing the oxygen reduction step (Pasco et al. 2005). Mediators can shuttle electrons from the bacterial ETC to an external electrode. For example, KFCIII is well known as a redox mediator that can couple with cellular respiration. The electrons released into the ETC through respiratory activity will be transferred to the mediator (e.g. KFC III) via membrane bound dehydrogenase enzymes that span the plasma membrane. This process leads to the accumulation of reduced mediator in solution. Transfer of electrons from the mediator to an electrode poised at a suitable voltage can generate a measurable current.

There are two significant advantages of SciTox™ over previous toxicity testing systems (Microtox®, *Spirillum volulans* motility inhibition, *Pseudomonas fluorescens* growth inhibition, and inhibition of respiration in a synthetic activiated sludge), by

using the synthetic medatior KFC III (Pasco et al. 2001). Firstly contrary to oxygen, the reduced form of the synthetic co-substrate has an initial background concentration of zero, thereby increasing the accuracy when measuring substrate conversion. Secondly, replacing oxygen with a synthetic co-substrate of higher solubility, a larger number of bacteria can be used and the incubation time is reduced.

The basic bacterial toxicity assay principle is based on comparing the deviation in a measurable current produced by healthy microbial cells relative to that produced by microbial cells exposed to a toxicant at a fixed concentration (Pasco et al. 2005).

2.3.4.1 Dose-response relationship & EC_{50}

Identification of dose-response relationship is one of critical steps in environmental risk assessment. It is used to display the response of experimental animals/microorganism cells to a range of toxicant concentrations (Figure. 2.3). In general, the accuracy of dose-response relationship could be improved by exposing the microorganisms to a large concentration range of toxicants.

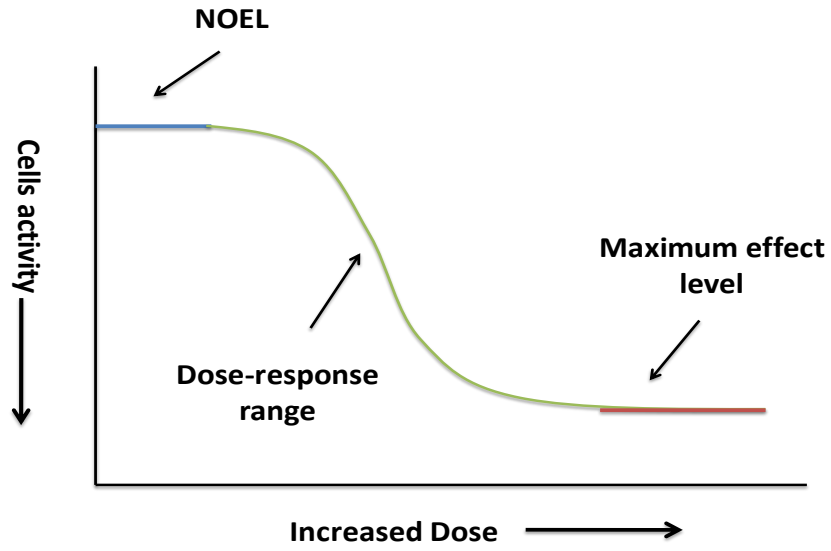

Figure 2.3. Dose-response curve

Dose-response relationship is termed as the change in effect when an organism is exposed to different levels of a stressor (e.g. chemicals). The dose-dependent manner could be described as: at a very low level (sub-inhibitory level) of toxins, there is no observed effect (NOEL) on an organism; at higher toxicant concentrations, activity of the organism decreases as dose is increased (dose-response range); and at extremely high toxin levels, it is fatal (maximum effect level) (Pasco et al. 2008).

However, the individuals of same species may have different resistance even when exposed to the same toxicant. Therefore, it is hard to identify the exact threshold dose. The NOEL is the highest exposure concentration at which there is no toxic effect on all individuals in a population, and at the maximum effect level, all individuals are affected. Thus, toxic effects are presented by using the index of measurable attributes such as EC_{50} (half maximal effective concentration). EC_{50} is the effective concentration at which 50% of individuals are affected in terms of mobility, growth inhibition, and respiratory inhibition. In the SciTox™ assay, EC_{50} is normally used as an index for toxicity determination (Pasco et al. 2008; Pasco et al. 2001).

2.3.4.2 Standard toxicants used in commercial toxicity test

Both 2,4- and 3,5- DCP are members of the chlorinated phenol family. Therefore, they share many chemical and physical properties with other chlorinated phenol compounds. They are both solids at room temperature, and the threshold of odour is very low (Ramamoorthy and Ramamoorthy 1997). Chlorinated phenols are man-made compounds that are mostly used as bactericides, fungicides and preservatives (Ramamoorthy and Ramamoorthy 1997). 2,4/3,5-DCP are commonly used as standard toxicants in commercial toxicity assays, including Microtox® (Kaiser and Palabrica 1991), ToxAlert® (dos Santos et al. 2002; la Farre et al. 2001), Micredox® (Pasco et al. 2008) and CellSense® (Farre et al. 2001).

2,4-dichlorophenol

2,4-DCP can be used directly for wood preservation and surface treatments for fresh-cut logs and lumber against sap-stain fungi and mould. It is also used for other industrial chemical synthesis. For example, it has been used to produce herbicide 2,4-

dichlorophenoxy (2,4-D), which acts as a plant growth hormone, and produce uncontrolled growth in target plants (Klaassen 2007).

2,4-DCP is a light-yellow crystal, with melting and boiling points of 41-44 ℃ and 209-210 ℃, respectively. There are two chlorine (Cl) groups at position 2 and 4 of benzene ring, with another OH group at position 1. Its solubility in water is very low. It is identified as a toxic chemical in European countries, and a highly toxic chemical in USA. Based on its lipophilic nature, it can be absorbed quickly in the gastro-intestinal tract (GIT) after ingestion. The target organs are blood and kidney. It also can cause skin and GIT burns.

3,5-dichlorophenol

3,5-DCP mainly acts as an intermediate for agrochemicals and other compounds, including fungicides, algaecides and bactericides, etc. It is a white crystal compound with melting and boiling point of 67-69 ℃ and 233 ℃ respectively. It is much more insoluble in water than 2,4-DCP. It can cause irritation of eyes, skin and respiratory system. Safety glasses and gloves are recommended when handling 3,5-DCP.

Both these dichlorophenols are widely used in the industrial field, and distributed in the environment. 2,4- and 3,5-DCP are potential harmful for human and animals. For example, 3,5-DCP had been identified as a hazard to aquatic environment (Zagorc-Koncan et al. 2002). However, some microorganisms have been reported to biodegrad chlorinated phenol compounds, including *Phancerochaete chrysosporium* (Valli and Gold 1991).

Copper (Cu^{+}/Cu^{2+})

Copper is one of the essential trace elements for microorganisms. Copper plays an important role in processing metalloproteins and act as co-factor for several enzymes (Gadd 1993; Nies 1999). Copper proteins are involved in a variety of biological process, including respiration, iron transport, oxidative stress protection, and blood clotting (Puig and Thiele 2002). Deficiency of these enzymes, or alteration in their activities, often causes disease states or pathophysiological conditions (Pena et al.

1999). However, higher Cu concentrations cause inhibition of growth or even death of microorganism (Ibrahim et al. 2008). The toxic effects of Cu on microorganisms include the displacement of essential ions, thereby obstructing functional groups of proteins, inactivating enzymes, producing hydroperoxide free radicals, and altering membrane integrity (Nies 1999). Therefore, Cu compounds have been widely used as algicides, fungicide, molluscides and acaricides in agriculture (Borkow and Gabbay 2005), and also as a food preservative (Ibrahim et al. 2008).

Copper ions undergo unique chemistry due to their ability to exist in distinct redox states, either oxidized as Cu^{2+} or in the reduced state, Cu^{+}. The Cu^{2+}-Nitrogen (N) bonds are normally inert while the bonds with oxygen donor ligands are more labile. Cu^{+} is considered a soft metal, and preferentially bonds with ligands such as sulfhydryl groups (Rensing and Grass 2003). Once inside the cells, additional proteins are required for sequestration and trafficking of Cu since intracellular free cations are toxic to the cells. The intracellular unbound Cu^{2+} can cause oxygen-radical damage (Freedman and Peisach 1989). Under anaerobic conditions, Cu appears to shift from Cu^{2+} to Cu^{+} oxidation state and become more toxic, possibly because Cu^{+} can diffuse readily through the cytoplasmic membrane (Beswick et al. 1976; Outten et al. 2001). Therefore, microorganisms need a homeostatic mechanism to avoid Cu toxicity.

Recently, two Cu-responsive regulatory systems have been identified (Rensing and Grass 2003). In the first system, a cation/proton antiporter complex, involved in export of metal ions, xenobiotics, and drugs, is encoded by genes *cusCFBA*. These genes are regulated by two genes *cusRS*, which form a sensor/regulator pair (Munson et al. 2000; Oshima et al. 2002). The second system, a *copA* gene encoded CopA protein, is a Cu^{+}-translocating P-type ATPase (Outten et al. 2000). The *copA* gene is regulated by CueR, a MerR-like transcriptional activator induced by Cu (Petersen and Moller 2000; Stoyanov et al. 2001). A model of Cu homeostasis mechanism can be summarised as: Cu^{2+} is only present in periplasm and Cu^{+} enters the cytoplasm by diffusion; Cu^{+} ions are effluxed from cytoplasm to periplasm by Cu^{+}-translocating P-type ATPase, CopA; Cu^{2+} or Cu^{+} ions in periplasm are then captured by CusF (a periplasmic Cu-binding protein), and form CusCBA complex, which is finally pumped out of outer membrane (Rensing and Grass 2003).

Silver (Ag^+)

The antibacterial effects of silver ions (Ag^+) have been well recognised (Klasen 2000; Silver and Phung 1996; Slawson et al. 1992). Silver has been used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds (Dibrov et al. 2002). Generally, the metallic Ag is relatively unreactive, and Ag^+ ions might be released when exposed to aqueous environments (Lansdown 2002).

In the presence of high Ag^+ concentration, the activity of several enzymes (containing mainly thiol groups) are inhibited (Jung et al. 2008; Park et al. 2009) because it reacts with electron donor groups, especially sulphydryl groups (Slawson et al. 1992). These effects may result in inhibition of cell division, damage to bacterial cell envelopes (Richards et al. 1984), and interruption of cell wall synthesis (Russell and Hugo 1994). In the presence of low Ag^+ concentration, the Na^+- translocating NADH: ubiquinone oxidoreductase (NQR) is one of the primary targets for Ag^+ ions. The Ag^+ ions have been shown to inhibit energy-dependent Na^+ transport in membrane vesicles of *Bacillus* sp. strain (Semeykina and Skulachev 1990) and also inhibit purified NQR of *Vibrio alginolyticus* (Hayashi et al. 1992). However, in a more recent study, the group clarified that NQR was not a specific target for low Ag^+ ion concentration. Low Ag^+ concentration has the ability to collapse the proton motive force through any Ag^+-modified membrane protein or perhaps through the Ag^+-modified phospholipid bilayer. Such effects can lead to a collapse of the membrane proton gradient causing a disruption to many cellular metabolism mechanisms which can ultimately lead to cell death (Dibrov et al. 2002).

Efflux pumps are composed of proteins either as ATPase or chemi-osmotic cation/proton antiporter (Nies 2003; Silver 1996), and Ag^+ has been associated with both of these mechanisms (Gupta et al. 1999). The Ag resistance conferred by the *Salmonella* plasmid pMGH100, contains a total of nine genes. Seven genes are named, in order, *silP*, *ORF105*, *silAB*, ORF96, *silC*, *silSR* and *silE* (Gupta et al. 2001) and two less-recognized,. The *silE* gene encodes a periplasmic Ag^+-binding protein (SilE). When SilE protein is loaded with Ag^+, five Ag^+ cations are retained per polypeptide (Lo et al. 2002). The *silCBA* genes determine a cavity/pore/funnel pathway for the Ag^+ ions to flow from the cytoplasmic region directly to the outer membrane protein (Murakami et al. 2002; Nies 2003). The gene *silP* encodes protein SilP which belongs

to the family of heavy metal resistance efflux P-type ATPases. SilP can pump Ag^+ ions from the cell cytoplasm to the periplasmic space (Rensing et al. 2000; Sharma et al. 2000). However, how periplasmic Ag^+ ions are removed is unclear. They might be removed through an unspecific outer membrane protein, or sequestered by SilE, sometimes followed by movement across the outer membrane via the SilCBA complex (Silver 2003). The Ag resistance system is transcriptionally controlled by two genes *silRS*. These two genes are presumed to be a two-component transduction pair, consisting of a membrane kinase sensor SilS and transcriptional regulatory responder, SilR (Silver 2003).

In bacterium *E. coli*, the Cu efflux system encoded by *copA* is also responsible for Ag^+ resistance (Su et al. 2011). CueR is known to be activated by the presence of Cu^+ and Ag^+ (Petersen and Moller 2000; Stoyanov et al. 2001). CueR contains a C-terminal metal binding region which is also conserved in MerR (Outten et al. 2000). However, CueR lacks a cysteine residue which is conserved at position 80 in MerR sequence. The difference in the metal binding domain of CueR might play a role in distinguishing between Cu/Ag (Outten et al. 2000).

2.4 Bacterial cell

Bacterial cells are surrounded by a cell wall. The cell wall plays an important role in maintaining the shape of the cell and preventing the cell from bursting when fluids flow into the cell by osmosis (Beveridge 1999). Peptidoglycan is one of the key components of the bacterial cell wall, which is made from polysaccharide chains and cross linked by the D-amino acid containing peptides (van Heijenoort 2001). The bacterial cell wall is surrounded by a secreted external structure, the capsule or slime-layer (Black 2008). A capsule only occurs in some bacteria (e.g. anthrax bacteria), whereas most bacteria have a slime-layer.

The cell membrane of bacteria forms the boundary between a cell and its environment. Cell membranes consist mainly of phospholipids and proteins (Black 2008). The bacterial cell membrane acts as a semi-permeable barrier to regulate the flow of substances such as water and nutrients into and out of the cell and as a surface where some important cellular reactions occur (Black 2008).

Bacteria can be divided into two main types; gram-positive and gram-negative. The Gram-staining method of bacteria was invented by Hans Christian Gram, in 1884 (Gram 1884). This method is based on the chemical and physical properties of bacterial cell walls (Bergey et al. 1994). The cell walls of gram-positive bacteria are mainly made of peptidoglycan, about 50%-90%. In contrast, the gram-negative bacteria cell walls generally contain about 10% peptidoglycan, and also have the additional outer lipid membrane which is separated by periplasmic space (Davies et al. 1983). In Gram staining (Beveridge 2001), bacterial cells take up crystal violet (CV). During decolourization, a decolourizer (95% ethanol or ethanol-acetone solution) dissolves the lipids in outer membrane of gram-negative bacteria. The CV cannot be trapped by the thin peptidoglycan layer of gram-negative cell that are washed from the cell along with outer membrane. In contrast, gram-positive cell becomes dehydrated from the treatment of a decolourizer, closing the pores of the cell wall during dehydration. The CV is trapped inside the thick peptidoglycan layer and the cell wall does not get decolourized. The bacteria used in this research are all gram-negative bacteria.

2.4.1 Culture and Growth

Most bacterial cells replicate by "binary fission" while some divide by budding. In binary fission, one bacterial cell divides into two identical cells (Thiel 1999). In budding process, a new cell develops from the surface of an existing cell and subsequently separates from the parent cell. These two identical cells are called clones. A cell population divides exponentially, forming a colony on a solid medium. All clones in the colony are derived from the original cell. Sometimes, the original bacterium is not a single cell, but a chain. However, the progeny also form a single colony in a solid medium (Thiel 1999).

In laboratory culture, bacteria grow exponentially, the number of bacterial cells can be calculated by 2^n (n is integer number; 0, 1, 2.....). The period between subsequent divisions is generation time. Fast growing bacteria such as *E. coli*, growing in optimal conditions may have a short generation time of only 15-20 mins (Thiel 1999).

In normal laboratory culture, four broad phases in bacterial growth can be identified (Black 2008); lag phase, log or exponential growth phase, stationary phase and the decline phase. In the lag phase, bacterial cells are newly inoculated into a media (agar plate or liquid media), and the cells need time to adjust their metabolism to the new conditions. There is no increase in cell number in this phase. The period of lag phase is dependent on the species of bacteria and also the media and conditions used. In the log phase, cell numbers increase exponentially. In closed systems, exponential growth leads to a decline in nutrients and a build up in growth-inhibiting substances. In the stationary phase, cells slow their metabolism and stop rapid growth. In the decline phase, bacterial cells lose their ability to divide and the cells start dying. To maintain cell viability, it is best to grow bacterial cultures to the early stationary phase (Thiel 1999).

Physical and chemical factors such as temperature, oxygen concentration and pH affect bacterial growth. Different bacteria have different growth optima for physical and chemical factors. The temperature used for cell culture depends on the type of bacteria. For example, most thermophilic bacteria grow at 50-60 ℃ (McHugh et al. 2006), whereas some such as Dictyoglomus *turgidas* have been reported to grow even at 86 ℃ (Kristjansson and Hreggvidsson 1995). *Staphylococcus aureus* grows rapidly at 48 ℃ (Fleury et al. 2009), but the optimal growth temperature of *E. coli* is only 37 ℃. For bacterial cell growth, three different critical temperatures are identified (Black 2008):

1. The minimum growth temperature: the lowest temperature required for cell division;
2. The maximum growth temperature: the highest temperature at which bacterial cells can divide;
3. The optimum growth temperature: the temperature for fastest growth rate and the shortest generation time.

Generally, bacteria can survive better at alkaline than acid pH (Thiel 1999), and most bacterial species in the environment, including *E. coli*, grow best in the pH range about 6-8. However, some species can survive in extreme conditions. *Acetobacter xylinum* is acid tolerant, and can grow even at pH 5 (Yamada and Yukphan 2008),

whereas the seawater *Pseudornona* spp. can grow well at pH 10.6 (Maeda and Taga 1980).

Bacteria can be mainly divided into four types (Black 2008), obligate aerobes, obligate anaerobes, facultative anaerobes and aerotolerant anaerobes. Obligate aerobes, such as *Pseudomonas*, require oxygen for respiration, whereas obligate anaerobes, such as *Clostridium botulinum*, cannot survive in the presence of oxygen. Facultative anaerobes, such as *E. coli*, can carry on aerobic metabolism when oxygen is present, but they shift to anaerobic metabolism when oxygen is absent. Aerotolerant anaerobes, such as *Lactobacillus*, can survive in the presence of oxygen but do not use it in their metabolism. The reason obligate anaerobes are sensitive to oxygen is that they lack enzymes to protect against the toxic oxygen molecules (Black 2008). For example, obligate anaerobes are killed not by gaseous oxygen but by a highly reactive and toxic superoxide. Superoxide is formed by oxidative enzymes. It can be converted into molecular oxygen and toxic hydrogen peroxide by enzyme superoxide dismutase. Hydrogen peroxide is further converted into water and molecular oxygen by the enzyme catalase. Obligate aerobes and most facultative anaerobes have both enzymes. Some facultative and aerotolerant anaerobes have superoxide dismutase but lack catalase. However, most of the obligate anaerobes lack both enzymes.

2.5 Microorganisms used in this research

Three different bacterial species, *A. calcoaceticus*, *E.coli* and *P.putida* were used in this research. All these are gram-negative bacteria, commonly used in the laboratory. *E. coli* and *P. putida* were chosen because they have been used in many studies and there is a pool of data available on their performance (dos Santos et al. 2002; Pasco et al. 2005; Tizzard et al. 2004). *A.calcoaceticus* was chosen because it has been shown to be quite stable under freeze-dried conditions (Idel'chik et al. 1983; Pirog et al. 1997).

2.5.1 *Acinetobacter calcoaceticus*

Acinetobacter species were originally reported by Brisou and Prevot in 1954 (Towner et al. 1991). *Acinetobacter* are strictly aerobic nonfermentative bacilli, which contain 17 validly named and 14 unnamed species (Dijkshoon 2008). These bacterial strains play a role in food spoilage and human disease. They are easy to isolate and culture in the laboratory. For most strains of *Acinetobacter*, the optimal growth temperature is about 30-35 ℃ but they also grow well at 37 ℃. Many are acid tolerant species, and can grow well at pH of 5.5-6.0 (Towner et al. 1991). *Acinetobacter* strains have the ability to remove phosphorus in activated sludge systems, and they are also effective in removing C_2-C_5 fatty acids and degradation of phenol (Adav and Lee 2008).

A.calcoaceticus are non-motile bacteria that have been used to remove oil contamination (Lal and Khanna 1996). Generally, *A. calcoaceticus* grows well in Nutrient Broth (NB) media, at a temperature range 16-40 ℃ (Du Preez and Toerien 1978) with optimum temperature at 37 ℃ (Obana and Nishino 1990).

2.5.2 *Escherichia coli*

E. coli is a well characterised food- and water-borne bacteria, which is ubiquitous in the mammalian GIT (Rankin 2008) and some strains can cause serious illness and even death. It was discovered by German paediatrician and bacteriologist Theodor Escherichia, in 1885 (Feng et al. 2002). *E. coli* is a gram-negative, facultative anaerobic and a non-sporulating bacteria. *E. coli* is widely used as the host of choice for the propagation, manipulation, and characterization of recombinant DNA, due to its easy transformability and genetic manipulation (Casali 2003). Almost all *E. coli* strains currently used in recombinant DNA experiments are derived from a single strain, *E. coli* K12 (Casali 2003). In this research, there are three *E. coli* mutated strains, *E. coli* Top10f' (tetracycline-resistant strain), *E. coli* K12 JW3564 (*selA* knock-out strain), and *E. coli* JW2283 (*nuoA* knock-out strain), used to carry genetically constructed plasmids.

2.5.3 *Pseudomonas putida*

P. putida is a rod-shaped, motile, non-spore forming, aerobic, gram-negative strain. *P. putida* has the ability to degrade plastics, and therefore has been used in environmental cleanup and pollution control (Shimao 2001). An outer membrane receptor protein, named pyoverdine, helps to transport the iron complex into the sideospores, where iron is used for metabolic processes and the oxygen acts as the final electron acceptor (Loper and Henkels 1999). *P. putida* can also regulate the degree of fatty acid saturation, in order to protect against environmental toxicants. For example, when the growth phase of *P. putida* shifts from exponential phase to stationary phase, the saturation of fatty acids and the membrane fluidity changes to a higher level (Hartig et al. 2005). *P. putida* strains do not generally cause human illness. However, they have been reported to cause disease in fish (Altinok et al. 2006). Generally, *P. putida* is easy to grow in the laboratory, and does not require any specific factors. *P. putida* can grow at the range of temperature 4-41 ℃, with optimal growth at 30-37 ℃ (O'Leary 1989).

2.6 Freeze drying

Freeze-drying, also known as lyophilisation, is a dehydration process used to preserve perishable materials. This technology has been widely applied in food industries, to improve the shelf-life of food stuffs. The basic principle of freeze drying is based on reducing the water activity of target materials. In recent years, freeze-dried biosensors have been used in environmental monitoring (Choi and Gu 2003).

Freeze-drying offers many advantages, including convenient to transport, easy to store and long shelf-life, etc. In the following section, the principle of freeze drying, storage and rehydration conditions will be briefly discussed.

2.6.1 Basic principle of freeze-drying and the Freeze Dryer

Freeze drying is a dehydration process used to remove the water in materials for long-term storage. It consists of two major steps; freezing the materials, and reducing the

surrounding pressure and adding more heat to allow the frozen water to sublime directly from solid phase to gas (Figure. 2.4).

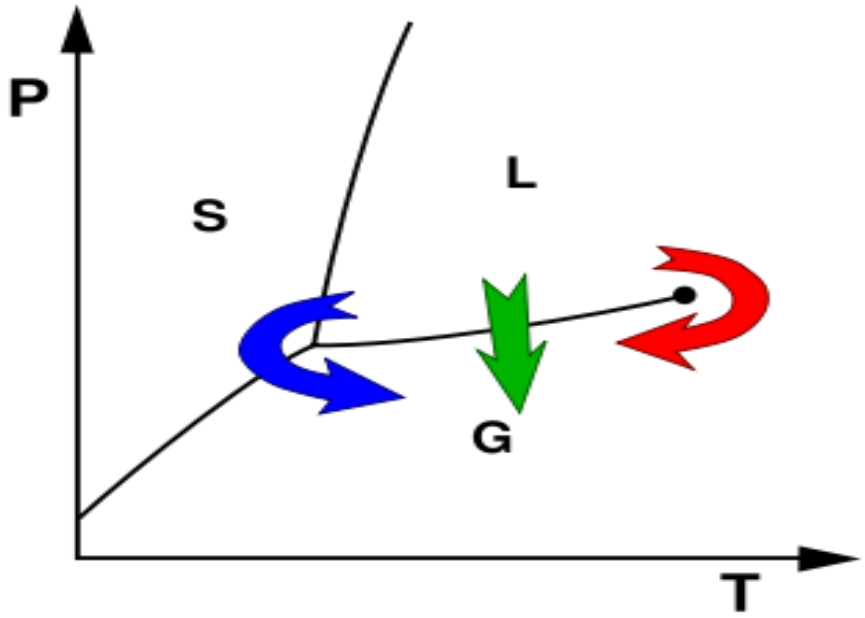

Figure 2.4. Phase-diagram (S-solid phase; L-liquid phase; G-gas phase); the direct transition from liquid to gas occurs when the temperature is between the triple point and the critical point (green arrow); freeze drying by bringing the temperature below the melting point (freezing) and reducing the pressures (blue arrow) so that a direct transition from the solid to gas takes place.

The first step of freeze drying is freezing. Normally the material is cooled below its eutectic point. The freezing phase is a critical step in the freeze drying process, because the large ice crystals formed may break the cell walls of bacteria and other microorganisms. The second step is drying. In this step, the low pressure and enough heat is supplied to materials for water to sublimate. The heat required can be calculated by using the Latent Heat Equation: Q = mL; Q (J) is represented by the energy required to change the physical phase (here means changing from solid to gas phase); m (Kg) is the mass of materials and L is the specific Latent heat for certain substances (Jg/Kg). After the primary drying process, up to 95% water of materials will be removed. In the secondary drying step, the unfrozen water inside of the material is removed. In this step, the pressure is also low but the T is higher than in the primary step to break any physico-chemical interactions which are formed

between water molecules and frozen materials. The water content in freeze dried material is between 1% and 4%.

2.6.2 Freeze drying factors

Freeze drying technology offers many advantages. However, the behaviour of bacterial cells may be altered on freeze drying. Freeze drying can damage cell membranes, increasing the membrane permeability (Castro et al. 1997). Moreover, loss of enzyme activities (e.g. ATP synthase) also occurs during freeze drying, due to either a change in membrane binding needed for enzyme function or direct damage to the enzyme itself (Castro et al. 1997).

The shelf-life of freeze dried bacterial cells varies with different strains (Fonseca et al. 2000), enterococci being more resistant than lactobacilli. Gram-positive strains *L. acidophilus* and *E. faecium* with 62.5% and 85.2% survival survive better than gram-negative strains *E.coli, P.putida* and *E.cloacae* with 42.6%, 33.5% and 50.8% survival (Miyamoto-Shinohara et al. 2006). Gram-positive bacteria are more resistant to freeze drying.

Sodium chloride (NaCl) can help bacterial cells to survive during freeze drying when it is added into the growth medium (Carvalho et al. 2003). Addition of some sugars into the growth medium, significantly increases the resistance resulting in longer shelf-life (Chervaux et al. 2000). For example, oligosaccharides can improve the viability of *lactobacillus reuteri* during freeze-drying (Schwab et al. 2007) and alginate and cryoprotecive sugars can improve the viability of lactic acid bacteria during the storage after freeze-drying (De Giulio et al. 2005). The OD also plays a role in cell viability during freeze drying (Schoug et al. 2006). Generally, the higher OD could increase cell viability on freeze drying. However, if the cell concentration is too high, it can be harmful to cells, due to an unbalanced osmotic pressure (Costa et al. 2000).

For optimal survival, freeze dried cells should be stored at low temperature, low pressure, low humidity and in the dark. High oxygen concentration and high level

water activity can also significantly reduce the survival rate of freeze dried bacterial cells (Kurtmann et al. 2009). However, if water activity is too low, the cells may die.

The optimal temperature for freeze dried bacteria cells are between 4 and -20 ℃. If freeze dried cells are stored at room temperature (e.g. 20 ℃), the viable cell count losses are approximately 100 times greater than those stored at 4 ℃ (Champagne et al. 1996). In another study (Miyamoto-Shinohara et al. 2006), the storage pressure was reported to be proportional to survival rate. Therefore, freeze-dried cells stored in a vacuum can also improve the survival rate.

The standard plate count is one of traditional methods are used for cell viability determination. This method is based on that only living-cells can divide on the agar plate to generate visible colonies (Black 2008). This measurement is expressed as the number of viable (living) cells per millilitre of culture. This step is used for evaluating the quality of freeze-dried cells in this research.

2.7 Summary

Biosensors have endeavoured to harness the ability of biological systems to recognize specific compounds, or classes of compounds to form the basis of analytical detect devices for many years. Biosensor technology has developed over the years and is currently used for the monitoring of a wide range of environmental toxic compounds (e.g. phenols, antibiotics, heavy metals, etc). Some of them are highly specific, such as enzyme-based biosensors. However, such type of biosensor has several drawbacks, such as short shelf-life of enzymes, and the limited interactions between environmental compounds and enzymes, etc.

SciTox^{TM} DTA assay is based on whole cell biosensors, offers several advantages over enzymes, including low cost, high stability and high adaptability. SciTox^{TM} could estimate the toxicity of a broad range of environmental chemicals, however, it lacks of specificity and sensitivity to specific analytes. Current advances in DNA technologies and genetic manipulation are providing improved microbial genetic information, facilitating the development of highly specific and sensitive microbial biosensors. To date only some groups have developed a genetically engineered

amperometric whole-cell biosensor to our knowledge (Lehmann et al. 2000; Shacham-Diamand et al. 2010; Tag et al. 2007). The sensor was based on the widely used *lacZ* reporter gene.

Electron transfer from bacteria to external acceptors is a biologically important phenomenon. This research aimed to find out the respiration-relevant reporter genes which are suitable for SciTox^{TM} amperometric biosensors. Ideally the performance of both specificity and sensitivity of SciTox^{TM} assay should be improved by using the genetically engineered whole-cell based biosensors (inducible-promoter gene fusion).

CHAPTER 3: STANDARD EXPERIMENTAL METHODS

3.1 Reagents

2,4-DCP (97% purity) and 3,5-DCP (99% purity) were purchased from Aldrich. All other chemicals were of analytical grade and purchased from BDH (Poole, UK).

All reagents (mediators and toxicants) used were prepared gravimetrically using a Sartorius A200S analytical balance, and made up to volume with room temperature sterile distilled water (dH_2O), and then autoclaved at 121 ℃ for 15 mins and stored at 4 ℃, which were sterilized under UV-light before use.

Potassium hexacyanoferrite (KFC III) (molecular weight 329.25 g mol^{-1}) was made to a final concentration of 250 mM with H_2O, covered with foil (to avoid light), and stored in a refrigerator at 4 ℃.

3.2 Buffers

Washing Buffer: weighed 10.62 mg l^{-1} of KH_2PO_4 and 21.25 mg l^{-1} of K_2HPO_4; made up to 1 l with dH_2O, autoclaved, and stored at room temperature.

Re-suspension Buffer: weighed 10.62 mg l^{-1} of KH_2PO_4, 21.25 mg l^{-1} of K_2HPO_4 and 7.46 mg l^{-1} of KCl; made up to 1 l with dH_2O, autoclaved, and stored at room temperature.

Polyethyleneglycol (PEG) solution: mixed 0.25 g of PEG with 100 ml of dH_2O, boiled, and cooled to room temperature. The solution was prepared fresh each time.

GTE (Glucose-Tris-EDTA) solution: consisted of 50 mM glucose; 25 mM Tris-Cl (pH adjusted to 8.0) and 10 mM EDTA (ethylenediaminetetraacetic acid; pH is 8.0). The solution was autoclaved and stored at 4 ℃ before use.

NaOH/SDS alkaline lysis solution: mixed 0.2 M of NaOH and 1% of SDS (sodium dodecyl sulphate), autoclaved, and stored at 4 ℃.

STE_{SDS} solution: 10 mM Tris; 1 mM EDTA; 100 mM NaCl and 1% SDS, autoclaved, and store at 4 ℃.

10 × TBE stock: 108 g Tris base; 55 g Boric acid; 40 ml of 0.5 M EDTA (pH 8.0), to 1 l with dH_2O, autoclaved, and stored at room temperature.

Herbert salt: consisted of 10.1 g MgO, 2 g $CaCO_3$, 53 ml of concentrated HCl, 5.6 g of $FeSO_4$, 1.4 g $ZnSO_4 \cdot 7H_2O$, 0.25 g of $MnSO_4 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$; 0.28 g $CoSO_4 \cdot 7H_2O$ and 0.06 g H_3BO_4, made up to 1 L with dH_2O, autoclaved, and stored at 4 ℃.

3.3 Standard toxicants and antibiotics preparation

400 mg l^{-1} 2,4/3,5-DCP: weighed 40 mg of 2,4/3,5-DCP, dissolved and made up to 100 ml with dH_2O. The solutions were prepared fresh for use.

100 mg ml^{-1} Amp (Ampicillin): weighed 100 mg of Amp, dissolved in 1 ml of dH_2O, sterilized the solution by passing it through a pre-rinsed 0.22um filter, and stored at -20 ℃.

50 mg ml^{-1} Kan (Kanamycin): 50 mg of Kan, dissolved in 1 ml of dH_2O, sterilized by passing through a pre-rinsed 0.22um filter, and stored at -20 ℃.

10 mg ml^{-1} Tet (Tetracycline): 10 mg of Tet, dissolved in 100% ethanol, mixed well, sterilized by filtering through a pre-rinsed 0.22um filter, and stored at -20 ℃.

3.4 Culture media

½ PC agar: 8.5g of m-plate count broth with 15 g agar, made up to 1 l with dH_2O, autoclaved, and stored at 4 ℃.

PC agar: 17g of m-plate count broth with 15 g agar, made up to 1 l with dH_2O, autoclaved, and stored at 4 ℃.

Nutrient Broth (NB): 8g of nutrient broth made up to 1 l with dH_2O, autoclaved, and stored at 4 ℃.

Davis Minimal Media (DMM): K_2HPO_4 (7g), KH_2PO_4 (2g), $(NH_4)_2SO_4$ (1g), glucose (1g), sodium (tri-) citrate (0.5g), $MgSO_4.7H_2O$ (0.1g), and made up to 1L with dH_2O, autoclaved, and stored at 4 ℃.

Basal Salt Medium (BSM): mixed K_2HPO_4 (4g), KH_2PO_4 (2g), (NH_4) SO4 (1g), yeast extract (0.5g), 2 ml of Herbert salt, made up to 1 l with dH_2O, autoclaved, and stored at 4 ℃.

LB-agar plate containing antibiotic: mixed 8.25 g of LB Miller (Difco^{TM} Luria-Bertani), 3 g of agar, made up to 300 ml with dH_2O autoclaved, and cooled to <50 ℃, and supplemented where appropriate with ampicillin (Amp) 100 μg ml^{-1}, kanamycin (Kan) 50 μg ml^{-1}, and Tet 10 μg ml^{-1}.

SOC medium: 2 g of bacto tryptone, 0.5 g of bacto yeast extract, 0.058 g of NaCl, 0.019 g of KCl, 0.203 g of $MgCl_2$, 0.247 g of $MgSO_4$, 0.36 g of glucose, dissolved in 100 ml dH_2O, autoclaved, sperated into 1 ml aliquots, and stored at -20 ℃ (Atlas 2004).

3.5 Culture of bacterial cells

3.5.1 Non-GMO cell culture

Three-step cell culture was prepared. Firstly, the bacterial cells were grown and maintained on agar plates, then a liquid pre-culture in duplicate, and finally the main-culture using the pre-culture as inoculum (Sambrook and Russell 2001).

3.5.1.1 Agar plate culture

Cells were streaked onto ½ PC/ PC agar plates, from -80 ℃ stock in freezer, wrapped agar plates by film and placed in the incubator, at 37 ℃ for 16 hours, stored cultured cells at 4 ℃ until required.

3.5.1.2 Liquid pre-culture

A single colony was selected and transferred from agar plate into a 100 ml conical flask which contained 40 ml of NB broth, grew in an orbital incubator at 37 ℃, 200 rpm for 16 hours, stored cells at 4 ℃ for less than 12 hours.

3.5.1.3 Main culture

2.5 ml of pre-culture was inoculated into a 500 ml flask with 250 ml bacterial grow media (DMM for both *E. coli* and *P. putida*; BSM for *A. calcoaceticus*), cells were grew in orbital incubator at 37 ℃, 200 rpm for 16 hours.

3.5.2 GMO cell culture

3.5.2.1 Agar plate culture

Cells were streaked onto LB agar plate and supplemented where appropriate with Amp 100 μg ml^{-1} (*E. coli* keio strain with plasmid transferred), Kan 50 μg ml^{-1} (*E. coli* keio mutants), and Tet (*E. coli* Top 10 f' strain) 10 μg ml^{-1}, plates were wrapped with film and maintained in an incubator at 37 ℃ for 16-19 hours, and cells were used as fresh (Song et al. 2012).

3.5.2.2 Liquid culture

A single colony was selected and transferred into a 100 ml conical flask containing 50 ml LB broth with 50 ul of appropriate antibiotic, cells were grew in an orbital incubator at 37 ℃, 200 rpm, for 12 hours (early-stationary culture of *E. coli* pSong8 and pSong9) and/or 8 hours (exponential culture of *E. coli* pSong10) (Song et al. 2012).

3.6 Cell harvest

Cells were pelleted by centrifugation at 12,851 rcf at room temperature for 5 mins, supernatant was decanted, cells were washed with 2 ml washing buffer, centrifuged cells at 12,851 rcf at room temperature for 5 mins and poured out the supernatant, repeated washing steps three times, and re-suspended cells with 2 ml of re-suspension buffer (Pasco et al. 2008).

3.7 Optical density measurement

Optical density (OD) also known as the index of light transmission and is also referred to as the absorbance. It is a measure of the light intensity at specified wavelength that passes through a sample relative to the intensity of the incident light. The higher OD of a material slower the movement of the wave through the material. Generally, OD is used as the measure of the concentration of a target solution. It is widely used in the biochemistry field, and can be calculated by using the formula (Equation. 1) (Voet et al. 2006).

Equation1: $A = -\log_{10}(I / I_0)$

A indicates the absorbance, OD; where I is the intensity of the transmitted light and I_0 is the intensity of the incident light.

In this research, a wavelength of 600 nm was used for OD measurement, and the fresh cultured bacteria were concentrated to an OD_{600} of 25 after harvest by using UNICAM 8625 UV/VIS spectrophotometer. An OD_{600} of 25 (1:40) represents the final concentration of the bacterial resuspension solution used for toxicity assay, and is about 4×10^9 bacterial cells per millilitre. In the SciTox^{TM} rapid mediated DTA assay, the lowest concentration of bacterial cells at OD_{600} should be at least 25 to obtain good results (Pasco et al. 2008). To prepare freeze dried bacteria, a higher concentration is used, OD_{600} of 60, which indicates about 10^{10} to 10^{11} bacterial cells per millilitre.

The concentration of bacterial cells measured by OD_{600} does not indicate the viability of cells. Therefore, a cell viability test needs to be carried out at the same time to

determine whether bacterial cells are still alive, and this is very important in study of freeze dried bacteria in toxicity assay.

Cells (genetically modified *E. coli* pSong8 and pSong9) used in specific SciTox^{TM} toxicity assay were adjusted to OD_{600} (with no dilution) of 4 – 6, *E. coli* pSong10 was not harvested by re-suspension buffer but maintained in media, The OD_{600} was adjusted to 0.5 - 0.8 (late-exponential growth phase) in SciTox^{TM} assay.

3.8 Preparing bacteria stock

750 ml of overnight culture was transferred into a 1.5 ml Eppendorf tube, mixed well with 750 ml of 50% glycerol, and stored at -80 ℃ in freezer (Sambrook and Russell 2001).

3.9 Plasmid mini-prep

1.5 ml of overnight culture was centrifuged for 20 seconds at 14,000 rcf in an Eppendorf MiniSpin Plus bench top centrifuge. The supernatant was removed completely (centrifuge, tipped out; centrifuge, then removed residual supernatant by pipett), and the pellet was re-suspended in 100 µl GTE and incubated for 5 mins at room temperature. 200 µl NaOH / SDS was then added and mixed gentlybefore placing back in ice for 5 mins. 150 µl of potassium acetate solution was then added and left on ice for 5 mins. Then the cultures were centrifuged for 3 mins at 14,000 rcf and the supernatant was transferred to a fresh tube. 1 volume of phenol and chloroform, or 2 volumes of phenol (chloroform) were added to dissolve the proteins, and then centrifuged for 5 mins at 12,000 rcf. After centrifugation, the solution seperates into two layers. The top layer (H_2O + DNA) was transferred to a fresh tube, and 2 volumes of 100% ethanol and 1/10 volume sodium acetate (salt resource) were added to precipitate the DNA molecules, incubated at room temperature for 2 mins, centrifuged for 5 mins at 14,000 rcf to precipitate plasmid DNA. The supernatant was tipped out, washed with 1 ml 70% ethanol, centrifuged at 14,000 rcf for another 3 mins. Ethanol was removed completely by air drying upside down in a laminar flow,

and re-suspended in 50 µl 10 mM tris. In some instances, used Invitrogen Plasmid mini-prep Kits (Purelink^{TM}, Invitrogen, and Carlsbad, USA).

3.10 Genome DNA isolation

150 µl of overnight culture was pelleted by centrifugation at 14,000 rcf for 3 mins, it was then re-suspended in 500 µl STE_{SDS}, and boiled for 5 mins. 500 µl of chloroform was added and mixed by inversion, centrifuged at 14,000 rcf for 5 mins, supernatant was transferred to a new tube, nucleic acids were precipitated with 1/10 volume 3M sodium acetate and 2 volumes ethanol, centrifuged at 12,000 rcf for 30 mins, washed with 0.5 ml of 70% ethanol, centrifuged at 14,000 rcf for 3 mins, ethanol was removed, then dried and re-suspended in 100 µl 10 mM Tris pH 8.0 (Sambrook and Russell 2001).

3.11 DNA quantification (by using Nanodrop-1000)

DNA quantification was by Nanodrop-1000 spectrophotometer (Thermo Scientific Ltd, Wilmington, USA) as per manufacturer's specifications.

3.12 PCR reaction

3.12.1 Hot start PCR (quick PCR system)

Table 3.1. Components of Hot-start PCR system

Reactants	Volume (µl)
10× NH_4^+ Buffer	5
25 mM Mg^+	2.5
Primer 1/2	1.25
20 mM dNTPs	1
DNA polymerase	0.25
DNA template	1
dH_2O	37.75
Total	50

One cycle of 95 ℃ for 10 minutes followed by 35 cycles of 95 ℃ for 30 seconds, 60 ℃ for 30 seconds, 72 ℃ for differential time (depends on size of PCR products, generally 1 min for 1 kilobase [kb]), followed by one cycle of 72 ℃ for seven minutes.

3.12.2 High Fidelity PCR (accurate PCR system-proof reading)

Table 3.2. Contents of High Fidelity PCR (HiFi-PCR) (Purelink^{TM}, Invitrogen, and Carlsbad, USA)

Reactants	Volume (µl)
PCR supermix	45
Primer 1/2	1.25
DNA template	1
Total	48.5

One cycle of 94 ℃ for 3 minutes, followed by 35 cycles of 94 ℃ for 30 seconds, 60 ℃ for 30 seconds, 72 ℃ for differential time (depends on the size of PCR products, generally 1 min for 1 kb), followed by one cycle of 72 ℃ for seven minutes.

3.13 PCR purification

PCR-amplified DNA fragments were purified with the Purelink^{TM} PCR purification kit (Invitrogen Ltd, Carlsbad, USA), as described in its protocol.

3.14 Restriction enzyme digestion

As shown in DNA digestion formula (Table. 3.3), incubated the reactants at 37 ℃ in a warm water bath for 1 hour.

Table 3.3. DNA digestion protocol

Reactants	Volume (µl)
Restriction enzyme	1
10× React$^{(X)}$ buffer (based on types of restriction enzyme; see appendix 10.1)	1
DNA fragment / plasmid	n (based on target DNA concentration; the amount of target DNA should be around 1000 ng)
dH_2O	25-n
Total	25

3.15 Agrose gel electrophoresis and gel extraction

Agarose gel electrophoresis:

0.7% agarose (25 ml) was prepared by mixing with 2.5ul dye, was poured into a tank, which was filled with 1 × TBE buffer, and the digested plasmid DNA was mixed with loading buffer (3 µl 6 × dye for 20 µl sample), DNA was loaded into the well of gel, 1 kb DNA ladder was loaded into another well of gel, and the gel was run for about 30 mins, at 90 volts, and the gel was then examined under UV-light (Sambrook and Russell 2001).

Gel extraction:

The band of interest DNA fragment was physically cut from agarose, and gel extraction by Purelink^{TM} Gel Extraction Kit (Invitrogen, Carlsbad, USA).

3.16 Ligation

The amount of plasmid vector and insert DNA fragment was determined by using the ligation formula (Equation 3): ng of insert DNA fragment= [ng (vector) × kb size (insert) / kb size (vector)] × [(3:1) (molar ratio)]. The optimal molar ratio used in ligation was 3 (vector): 1 (insert) (short insert <1 kb) or 1 (vector): 1 (insert) (large insert >1 kb), and ligation was preferred by incubating the reactants together (Table. 3.4) in 1.5 ml Eppendorf tube at room temperature overnight (Sambrook and Russell 2001).

Table 3.4. Ligation details

Reactants	Negative control (µl)	Sample (µl)
Vector	n (~150 ng)	n
Insert	-	m (calculated by Equation. 3)
T4 ligase	1	1
5 × T4 ligation buffer	2	2
dH_2O	10-n	10-n-m
Total	10	10

3.17 Preparation of Electroporation competent *E. coli* cells

250 ml LB broth was poured into a 500 ml flask, 2.5 ml overnight pre-culture was inoculated and incubated at 37 ℃, 200rpm. The OD_{600} (1:1) was tested to about 0.5-0.8 (normally took about 3 hours), and the 500 ml flask was placed in an ice-bath immediately, and cooled for 30 mins. All tubes (AXYGEN® 50 ml screw cap tubes & 1.5 ml Eppendorf tubes) were incubated in ice-bath and cooled in 10 % glycerol at 4 ℃, until required. The centrifuge was set at 0 ℃, 3,200 rcf, with accel˜= 6˜, and centrifuged for 20 mins. 50 ml of ice cold cells were poured into 50 ml screw cap

tubes and harvested by centrifugation. 15 ml of ice cold 10 % glycerol was poured into each tube, and re-suspended gently, until all cells were fully re-suspended, the tube was then filled up to 50 ml with 10 % glycerol, and the cells were washed by centrifugation. The washing step was repeated three times. Finally, the cells were re-suspended in 1-2 ml of 10 % glycerol, then aliquoted 40 µl each into 1.5 ml Eppendorf tubes, and stored at -80 ℃, until required (Sambrook and Russell 2001).

3.18 Clone plasmid into electroporation competent *E. coli* cells

The electroporation competent cells (40 µl) were thawed at room temperature, and then placed on ice. 0.2 cm sterile cuvettes were placed on ice along with the gene pulsar slide. 1-2 µl of ligated DNA samples were pipetted into competent cells, and mixed well with stirring tips and incubated for 1 min. The gene pulsar was set to Bacteria/Ec2 and incubated DNA sample was transferred into a cuvette. Cuvette was placed in slide and into the chamber, pulsed once, the cuvette was removed and 1ml of SOC medium was quickly added (Sambrook and Russell 2001).

3.19 Electroporation competent cell recovery

The plasmid cloned bacterial cells were placed in the orbital incubator at 37 ℃, 200 rpm for 1 hour. About 100 µl of cells were poured onto appropriate antibiotic containing LB agar plate. Plate was sealed by film and incubated at 37 ℃ for 18-20 hours (Sambrook and Russell 2001).

3.20 Blue-white colour assay

40 µl of 20 mg ml^{-1} X-gal and 50 µl of 0.1 M IPTG were poured onto appropriate antibiotic coated LB agar plate. The plate was dried for 1-2 hours that allowed chemicals (X-gal and IPTG) to be well absorbed, the transformation mixture /bacteria from stock were placed onto LB agar plates and dried. The plates were incubated

overnight (16-18 hours) invertely in a 37 ℃ incubator, and then left into refrigerator at 4 ℃ until required. The blue precipitate was clearly visible after 2 hours (Sambrook and Russell 2001).

3.21 Conjugation

A single colony was selected from each of two overnight petr-dish cultures, and then transferred each colony into a separate flask with 50 ml of LB containing appropriate antibiotics. The cells are grown for overnight at 37 ˚C, 200 rpm. 10 ml of each overnight flask-culture was transferred together into a new empty flask, and incubated together at 37 ˚C, 200 rpm for 4 – 5 hours. The culture mixture was then diluted to 1:100 with LB broth. Spread 100 ul from 1:100 dilutions onto an antibiotics coated LB agar plate. The agar plate was then placed into a 37 ˚C incubator for overnight (Raya and Jabri 2008).

3.22 SciTox™ toxicity assay

3.22.1 Non-specific SciTox™ toxicity assay

200 µl DTA assays were conducted in 96-well plate, each well contained 119 µl of substance, 44 µl of 250 mM mediator (KFC III), and 37 µl of cells (See Table. 3.5 for details) (Pasco et al. 2008).

Table 3.5. 200 ul Direct Toxicity Assay (DTA) (original conc. of 2,4/3,5-DCP was 400 mg l^{-1}).

Target concentration (mg/L)	Take from toxicant stock (µl)	Water for dilution (µl)	Mediator (250mM KFC III)	Cells (µl)	Total (µl)
Control	0.0	119.0	44	37	200
10	5.0	114.0	44	37	200
20	10.0	109.0	44	37	200
30	15.0	104.0	44	37	200
40	20.0	99.0	44	37	200
50	25.0	94.0	44	37	200
60	30.0	89.0	44	37	200
70	35.0	84.0	44	37	200
80	40.0	79.0	44	37	200
90	45.0	74.0	44	37	200
100	50.0	69.0	44	37	200

The 96-well plate was covered with foil, was then incubated in orbital incubator at 37 ℃, at 200 rpm for a one hour single step incubation. The 200 µl of reagents were transferred to clean Eppendorf tubes. The reaction was terminated by centrifuging at 11,000 g for 3.5 mins. The pellets were removed, and 170 µl of supernatant was transferred to clean Eppendorf tubes. Samples were stored at 4 ℃ until required for electro-chemical analysis.

3.22.2 Specific SciTox™ toxicity assay

The SciTox™ assay was performed in 24-well microtest plates. 1 ml of washed cells (for *E. coli* [pSong9]) and 1 ml of cultured cells (for *E. coli* [pSong10]) or 925 µl of cells plus 75 µl of 252 mM lactose (for *E. coli* [pSong8]), were incubated with 1 µl of variable concentrations of Tet. The plates were incubated at 37 ℃, 200 rpm for 2 h. Next 300 µl of 250mM KFCIII was added to each well. The microtest plates were

wrapped in aluminium foil to exclude light and incubated in an orbital shaker at 37 ℃, 200 rpm for 1 h. Assays were terminated by transferring the contents of each well into 1 ml Eppendorf tube and centrifuged at 12851 rcf for 4 min to pellet cells. The supernatants were transferred into clean Eppendorf tubes and then stored at 4 ℃ until microelectrode amperometry analysis (Song et al. 2012).

3.23 Electro-chemical analysis

Limiting current microelectrode amperometry, 3-electrode system comprising a working, a reference and an auxiliary electrode, were used to measure the quantity of reduced mediator produced during the microbial incubation. The reference and auxiliary electrodes were directly immersed in phosphate saline buffer supporting electrolyte, contained within a cell vial (BAS MF-1082). The 1 ml sample was confined in a separate working-electrode compartment (BAS MF-2031, 2 ml mini-cell). A 200 µl sample was injected into BAS MF-2031 2ml minicell. The working-electrode was poised at +450 mV relative to the Ag/AgCl reference electrode and anodic current obtained 10 s after imposition of the applied potential was taken as the limiting current value. After each analysis, the working-electrode was re-polished and the BAS MF-2031 minicell rinsed in dH_2O and dried using lint-free tissue (Pasco et al. 2008).

3.24 Data analysis

For electrochemistry analysis, the current produced was measured and the cell activity was determined when exposed to different toxicant concentrations using the EC_{50} value as toxicity index (Equation 2) (Pasco et al. 2008).

EC_{50} was measured based on both calibration curve (dose-response curve) and calculation.

Equation2:

$$\%\ Activity = \left[\frac{i_{lim}(sample)}{i_{lim}(control)}\right] \times 100\%$$

The i_{lim} (*sample*) is the amperometric limiting-current for a sample spiked with standard toxicant; i_{lim} (*control*) is the limiting-current of the negative control.

Data analyses:

The computer programme, "SigmaPlot" was used for data analysis and establishing a dose-response curve, and calculating the standard error (SE) and EC_{50}.

Specific toxicity assay:

Three different sensors were exposed to a wide Tet concentration range, and the dose-response was profiled using Microsoft Excel. Then attention was restricted to the linear dose-dependent response range and the experiments were repeated to envelope only the linear response range of the sensors. Both detection limit and quantification range of Tet with three sensors were determined based on standard linear calibration curve predicted by Excel.

Detection Limit

Several approaches for determining the detection limit are possible, depending on whether the procedure is non-instrumental or instrumental. Approaches other than those listed below (Group 2005).

1. Based on visual evaluation:

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The detect limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

2. Based on signal-to-noise

This approach can only be applied to analytical procedures which exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

3. Based on the standard deviation of the response and slope

The detection limit (DL) may be expressed as:

Equation 4: $DL = 3.3\sigma/S$

Where σ= the standard deviation of the response

S= the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example:

A. Based on the standard deviation of the blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

B. Based on the calibration curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

4. Recommended data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatogram is considered acceptable for justification.

In case where an estimated value for detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

3.25 Freeze-drying

Bacterial cells were grown as described in (**section 3.5.1)**. The bacteria were harvested and concentrated to OD_{600} of 60 with appropriate dilution (1:60). The total volume of concentrated bacterial cells required was 50 ml. 100 ml PEG solution (a cryoprotectant to prevent the formation crystals during freezing process, and protect bacterial cell membrane) was mixed, and incubated for 30 mins at room temperature. 1.2 ml each of the mixture was transferred into each well of 24-well plate, and the plates were frozen at -80 ℃ overnight. The frozen plates (with the lids removed) were placed into the trays of Labconco Freeze drier, and freeze-dried for about 24 hours. The freeze-dried bacterial cells were transferred into a bottle and stored in a desiccator at 4 ℃, until required.

Cells were resuscitated by adding 280 ul of phosphate buffer saline solution into each well. Sealed the plates with parafilm and rehydrated them in an orbital incubator at 37 ℃, 200 rpm for 24 hours. After rehydration, the cells used in toxicity assays followed an identical protocol as that described for the fresh cells.

CHPATER 4: UNMODIFIED SCITOX™ RAPID DTA ASSAY USING FREEZE-DRIED CELLS

4.1. Introduction

The SciTox™ toxicity assay is a commercially available, whole cell microbial assay that measures toxicity through inhibition of bacterial respiration (Tizzard et al. 2004) and is currently used to detect and quantify wastewater toxicity. It is a rapid catalytic microbial method in which the natural co-substrate, oxygen, is substituted by the redox mediator potassium hexacyanoferrate KFC(III) (Pasco 2005). Electrons derived from oxidation of the substrate are released into the electron transport chain and ultimately to the external mediator KFC(III). This process leads to an accumulation of reduced mediator, KFC(II) in solution. Transfer of electrons from the mediator to an electrode poised at a suitable voltage can generate a measurable current and is used to quantify the magnitude of respiration inhibition and indirectly the toxicity.

Recently, freeze-dry technology has been widely studied in toxicity assays because it offers convenience including ease of storage and transport (Gaiek et al. 1994; Leslie et al. 1995; Perry 1995) and a stable biosensing tool to monitor and detect toxicity (Choi and Gu 2003; Shin et al. 2005). Many factors significantly affect freeze-dried cell stability. These include the toxicity of cryoprotectants (Kuleshova et al. 1999), storage temperature (Champagne et al. 1996), water activity (Kurtmann et al. 2009) and bacterial strain (Miyamoto-Shinohara et al. 2006). To our knowledge, there are no reports on the shelf life of freeze-dried cells used in toxicity assays.

In this research, the shelf life of freeze-dried cells affected by three different factors: cryoprotectant type, bacterial strain, and storage temperature, was studied using the SciTox™ DTA. To do this, three bacterial strains, *Acinetobacter calcoaceticus* (*A. calcoaceticus*), *Escherichia coli* (*E. coli*) and *Pseudomonas putida* (*P. putida*) were chosen because they have been used previously in toxicity studies and there is some data available on their performance (dos Santos et al. 2002; Pasco 2005; Tizzard et al. 2004). Two chloride phenol compounds, 2,4-dicholorophenol (2,4-DCP) and 3,5-dichlorophenol (3,5-DCP), were used as reference toxicants as they have been

commonly used in many commercial toxicity assays including Microtox® (Kaiser and Palabrica 1991), ToxAlert® (dos Santos et al. 2002) and CellSense® (Farre et al. 2001).

The bacterial strains were prepared in two forms, as fresh cultured and freeze-dried cells. Each freeze-dried bacterial strain was prepared with two cryoprotectants (polyethylene glycol [PEG] and sucrose/Tween 80). PEG is a well known cryoprotectant used in freeze-dried assays. However, there are reports that it is toxic and reduces cell viability (Gayle et al. 2006; Kuleshova et al. 1999). Sugars, such as glucose and sucrose are relatively non-toxic compared to PEG (Gayle et al. 2006; Kuleshova et al. 1999) and give good cell viability as a cryoprotectant (Shin et al. 2005). Moreover, 12% sucrose exhibited the best cell viability compared to all other tested single sugar or sugar mixtures when used in freeze-dried cells (Shin et al. 2005). Tween 80 is a polyethylene sorbitol ester with little or no anti-bacterial activity (Dawson et al. 1986). It has been reported that sucrose combined with 0.2% Tween 80 significantly increases freeze-dried cell viability (Choi and Gu 2003).

Storage temperature has been reported to play an important role in the viability of freeze-dried cells, the lower the storage temperature, better the cell viability (Champagne et al. 1996). Other factors such as storage pressure also play an important role in cell viability, and freeze-dried cell survival rate is significantly improved by storing the cells in a vacuum (Miyamoto-Shinohara et al. 2006). However, we sought to study the shelf-life of freeze-dried cells under laboratory conditions (normal atmospheric pressure and stored in a desiccator) that could be easily replicated in a research or a commercial laboratory. Based on this, the efficiency of two different storage temperatures (4 ˚C and -20 ˚C) was evaluated in this study.

In order to evaluate the effects of the three factors (cryoprotectant type, bacterial strain, storage temperature) on shelf-life stability of the freeze-dried strains, the dose responses to two standard toxicants (2,4- and 3,5-DCP), were measured by SciTox™ assay of three different freeze-dried bacterial strains, each with two different treatments (PEG and sucrose/80), and two different storage conditions (4 ˚C and -20 ˚C). This measurement sequence was repeated over three storage periods (at 1, 2 and 3 months) by comparing the dose-response to standard toxicants of resuscitated freeze-dried cells relative to their freshly cultured counterparts. To better understand

the effect of freeze-drying process on the viability of freeze-dried cells and the efficiency of cryoprotectants during the freeze-drying, the responses of freeze-dried cells (resuscitated immediately following freeze-drying) at zero-time to standard toxicants were measured as controls.

4.2. Methods

Chemicals: 2,4-DCP (97% purity) and 3,5-DCP (99% purity) were purchased from Aldrich. All other chemicals were of analytical grade and purchased from BDH (Poole, UK). All reagents (mediators and toxicants) used were prepared gravimetrically using a Sartorius A200S analytical balance, and made up to volume at room temperature with sterile distilled water. Potassium hexacyanoferrate (KFCIII), the mediator, was made up fresh and wrapped in aluminium foil and stored in the dark until use.

Microbial culture: *A. calcoaceticus*, *E. coli* and *P. putida* were maintained on Plate Count Agar (PC agar) (Atlas 2004) at 4 ˚C. Pre-cultures were prepared by inoculating a single colony from the agar plate into 20 ml of broth in a 100 ml conical flask. *E. coli* pre-cultures were grown in a Davis Minimal Media (dipotassium hydrogen orthophosphate 7 g/l, potassium dihydrogen orthophosphate 2 g/l, ammonium sulphate 1 g/l, glucose 1 g/l, sodium citrate 0.5 g/l, magnesium sulphate 0.1 g/l) at 37 ˚C. *A. calcoaceticus* and *P. putida* were grown on Difco m-plate Count Broth at full recommended strength (15 g/l) and at 37 ˚C. All pre-cultures were grown aerobically on an orbital shaker (200 rpm) for 16 hours (over-night) and stored at 4 ˚C until required.

A 5% inoculation of the appropriate media was carried out using the pre-cultures. Flasks were placed on an orbital shaker (200 rpm) overnight (16 hours) and incubated at 37 ˚C for all three strains. Cells were harvested by centrifuge (Eppendorf 5810R centrifuge) at 10 000 × g for 5 min at room temperature. Cells were washed three times in phosphate buffer (0.05 M K_2HPO_4/KH_2PO_4 pH 7.4, 0.1 M KCl). The cell

concentration was adjusted to an absorbance ($A_{600\ nm}$) of between 20 and 25, and the cells were stored at 4 ˚C until required, typically less than 30 min.

Freeze-dried cells preparation: Each of three bacterial strains was prepared into two freeze-dried forms (PEG and sucrose/Tween 80).

Preparation by using PEG: 100 ml of 2.5 g/l PEG solution was made up using sterile distilled water (dH_2O) and boiled for 5 min. The PEG solution was cooled to room temperature prior to mixing with 50 ml of cells (with absorbance of $A_{600\ nm}$ about 60). Once mixed, the solution was left to stand to ensure that the cryoprotective media had sufficient time (approx 10 mins) to thoroughly permeate the cells of the suspension. After standing for a desired time period (10 mins) 1.5 ml of the mixture was added into each well of 24-well plates (Coasta 3524) and stored overnight at -80 ˚C. The frozen cells were then freeze-dried using a Labconco freeze drier and the whole process took about 24 hours.

Preparation by using 12% sucrose and 0.2% Tween 80: 500 ml of 50% (weight/volume) sucrose stock solution was made up using dH_2O, and sterilized by passing through a 0.22 µm filter. The sucrose stock solution was exposed to UV light for 5 mins. 25 ml of 50% sucrose, 0.2 ml of Tween 80 were transferred and mixed with 80 ml of cells (with absorbance of $A_{600\ nm}$ about 60). This solution was left to stand to ensure that the cryoprotective media had sufficient time (approx 10 mins) to permeate through the cell suspension. After 10 mins, 1.5 ml of the mixture was added into each well of the 24-well plates (Coasta 3524) and stored overnight at -80 ˚C. The frozen cells were then freeze-dried for 24 hours in a Labconco freeze drier.

Storage conditions: Once cells were freeze-dried, each form (either PEG or sucrose/Tween 80) of freeze-dried cells was aliquotted into two separate 50 ml glass bottles. The bottles were sealed with parafilm and packaged into a plastic bag which contained the dessicant, sealed with tape, and placed in two separate desiccators, each of which was stored at either 4 ˚C or -20 ˚C.

Freeze-dried cells resuscitation: 0.04 g freeze-dried cells were mixed with 400 µl 0.05 M PO_4/0.1 M KCl buffer in a 5 ml screw cap tube. The cells were rehydrated by shaking at 200 rpm and 37 ℃ for 24 hours. Once rehydrated, the cells were adjusted to $A_{600\ nm}$ 25 before use.

SciTox^{TM} toxicity DTA assay: The toxicity of both standard toxicants was examined at no less than 10 different concentrations with each bacterium. The SciTox^{TM} toxicity assays were performed in 96-well plates. Each well contained 119 µl of toxicant at variable concentration, 44 µl of 250 mM KFC(III), and 37 µl of cells. The 96-well plates were wrapped with aluminium foil and incubated on an orbital shaker at 200 rpm and 37 ˚C for 1 hour. Assays were terminated by transferring the contents of each well into 1ml Eppendorf tube and centrifuging at 10, 000 × g for 4 min to pellet the cells. The supernatants were then transferred into clean Eppendorf tubes, and stored at 4 ˚C until microelectrode amperometry analyses.

Data analysis: When SciTox^{TM} is operating in toxicity mode, the current (required to re-oxidise the reduced mediator) produced by healthy cells was compared to the current produced by cells subjected to a fixed toxin concentration. By comparing the ratio of the electrochemical signal obtained in the presence and absence of a toxin, it was possible to obtain an index of respiratory inhibition, % Inhibition, was shown in the Equation below, where i_{lim}(sample) is the response of the test substance and i_{lim}(control) is the control response (as limiting current, nA).

$$\%\ Inhibition = 100 - [100 * \left(\frac{i_{lim}(sample)}{i_{lim}(control)}\right)]$$

The dose-response relationship curves were processed using "SigmaPlot 11" (Systat Software). EC_{50} and other statistical values (such as P value) were also generated by this programme. The EC_{50} calculated by "SigmaPlot 11" is the toxicant concentration corresponding to half way between the no observed effect level (100% respiratory activity) minus the signal saturation level (minimal respiratory activity).

Cell viability test: Cell viability was evaluated based on petri-dish plate count. All cells were harvested and adjusted to $A_{600\ nm}$ 25. 100 µl of cells with appropriate

dilutions was spread onto a PC agar plate. The plates were then placed in a 37 ˚C incubator for overnight culture. The cell viability was calculated by the number of colonies of freeze-dried cells divided by the number of colonies of their fresh counterparts.

4.3. Results

4.3.1. Fresh cells toxicity assay

Two dichlorophenols 2,4-DCP and 3,5-DCP were chosen as the reference toxicants as their toxicities have been widely reported using other toxicity assessment assays (dos Santos et al. 2002; Farre et al. 2001; Kaiser and Palabrica 1991). Three fresh cultured bacterial strains *A. calcoaceticus*, *E. coli*, and *P. putida* were chosen to provide benchmarks for assessing the dose-response of freeze dried cells when exposed to matching concentrations of reference toxicants.

The dose-response relationships of the three fresh-cultured bacterial cells determined by using SciTox^{TM} DTA assay when exposed to variable concentrations of standard toxicants are shown in Figure 4.1. The entire dose-response manner of three bacterial strains to each standard toxicant could be described as: at the low toxicant concentration (< 10^0 mg/l) there was no significant respiratory inhibition effect detected (no observed effect level; NOEL); at toxicant concentrations between 10^0 and 10^3 mg/l the cell's respiratory activity declined progressively as the toxicant concentration increased (dose-dependent response); and at high toxicant concentrations (> 10^3 mg/l), the cells showed no further loss of respiratory activity (signal saturation level).

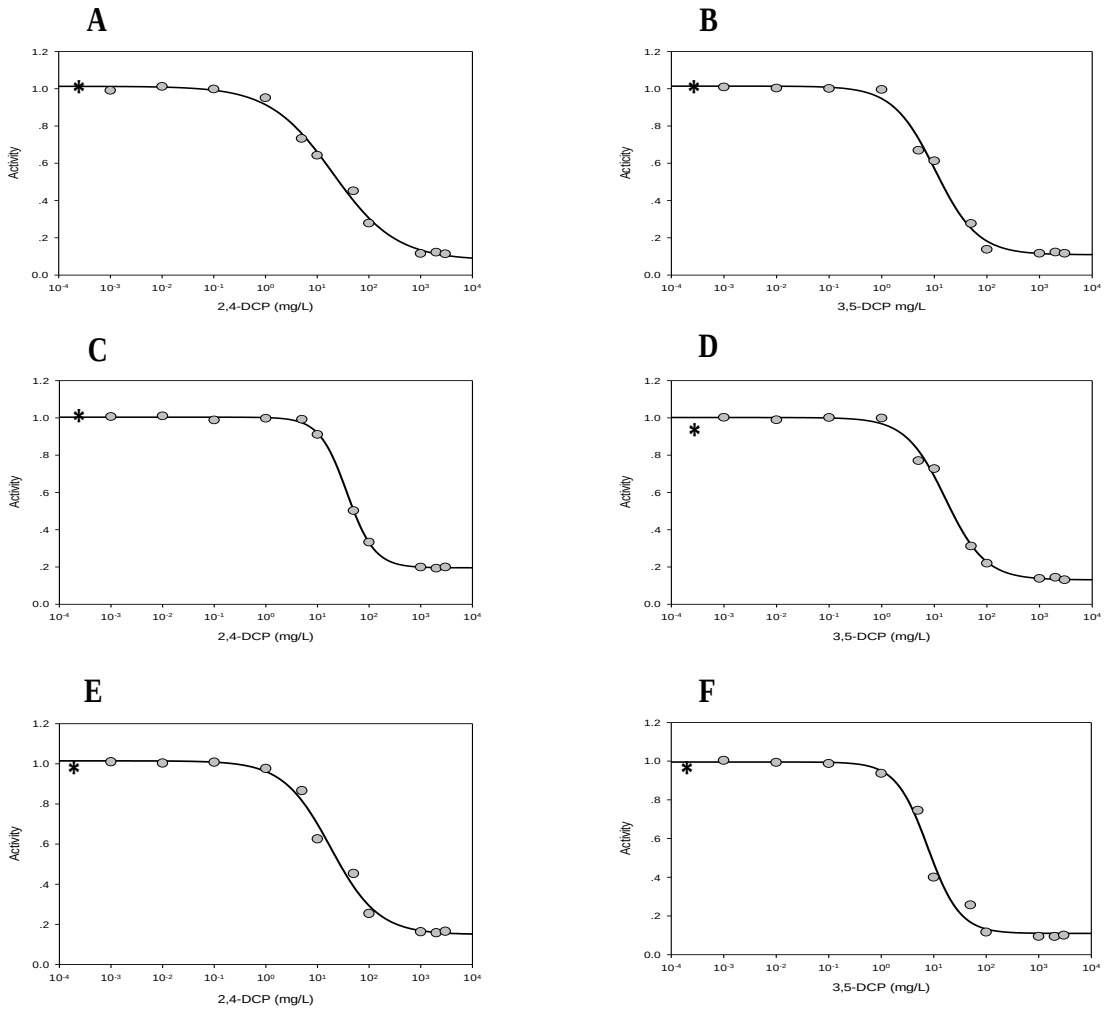

Figure 4.1. Dose-response of fresh cultured bacterial cells, when exposed to two standard toxicants. Diagrams A and B show the dose-response of *A. calcoaceticus* to 2,4-DCP and 3,5-DCP, respectively. Diagrams C and D show the dose-response of *E. coli* to 2,4-DCP and 3,5-DCP, respectively. Diagrams E and F show the dose-response of *P. putida* to 2,4-DCP and 3,5-DCP, respectively. * is negative control (zero toxicants). n = 3, $P < 0.001$.

The EC_{50} values are listed in Table 4.1. Of the three bacterial strains, *P. putida* exhibited the lowest EC_{50} to both 2,4-DCP and 3,5-DCP indicating the sensitivity of this bacterium to the two toxins, whereas *E. coli* showed the highest EC_{50} values. Moreover, the EC_{50} of bacterial cells to 3,5-DCP was lower than the EC_{50} to 2,4-DCP (Table 4.1) showing that 3,5-DCP was more toxic than 2,4-DCP.

4.3.2. Zero-time freeze-dried cells toxicity assay

Following freeze-drying, both forms (PEG, and sucrose/Tween 80) of the three freeze-dried bacterial strains were resuscitated and used as the biocomponent (controls) in the SciTox^{TM} toxicity assay. The experimental procedure and toxicant concentrations were identical to those described for the fresh cells.

Dose-dependent activity of the zero-time PEG pre-treated freeze-dried cells to the two standard toxicants (Figure. 4.2) in SciTox^{TM} toxicity assay was similar to the freshly cultured counterparts (Figure. 4.1). The EC_{50} values of PEG pre-treated freeze-dried cells were lower than the EC_{50} of fresh cells to both standard toxicants (Table 4.1) but remained within the estimates of error.

Sucrose/Tween 80 pre-treated freeze-dried cells also responded to standard toxicants in a similar dose-dependent manner (Figure. 4.2), compared to their fresh counterparts (Figure. 4.1). However, the EC_{50} values of the sucrose/Tween 80 pre-treated freeze-dried cells were much higher than the PEG pre-treated cells to the two standard toxicants, compared with their freshly cultured counterparts (Table 4.1).

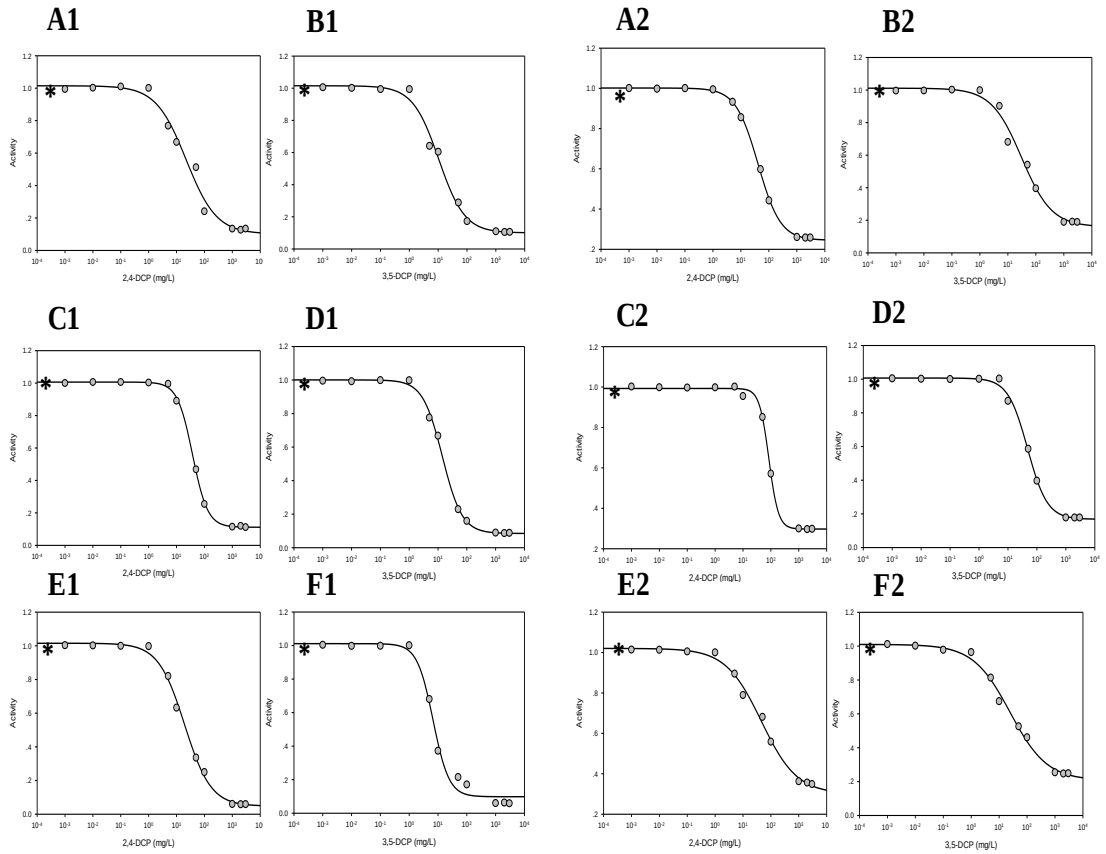

Figure 4.2. Dose-response of zero-time freeze-dried and two different cryoprotectants treated freeze-dried bacterial cells, when exposed to two standard toxicants. Diagrams A1 and B1 show the dose-response of PEG treated *A. calcoaceticus* to 2,4-DCP and 3,5-DCP, respectively. Diagrams C1 and D1 show the dose-response of PEG treated *E. coli* to 2,4-DCP and 3,5-DCP, respectively. Diagrams E1 and F1 show the dose-response of PEG treated *P. putida* to 2,4-DCP and 3,5-DCP, respectively. Diagrams A2 and B2 show the dose-response of sucrose and Tween 80 treated *A. calcoaceticus* to 2,4-DCP and 3,5-DCP, respectively. Diagrams C2 and D2 show the dose-response of sucrose and Tween 80 treated *E. coli* to 2,4-DCP and 3,5-DCP, respectively. Diagrams E2 and F2 show the dose-response of sucrose and Tween 80 treated *P. putida* to 2,4-DCP and 3,5-DCP, respectively. * is negative control (zero toxicants). n = 3, $P < 0.001$.

Table 4.1: EC_{50} values of fresh cultured cells and two different forms of their zero-time stored freeze-dried counterparts to 2,4-DCP and 3,5-DCP toxicants tested in SciTox^{TM} toxicity assay (n = 3, P < 0.001).

Bacterial strains	**EC_{50} values (mg/l)**					
	2,4-DCP			**3,5-DCP**		
	Fresh	**0-time freeze-dried**		**Fresh**	**0-time freeze-dried**	
		PEG	**Sucrose + Tween 80**		**PEG**	**Sucrose + Tween 80**
A.calcoaceticus	25.1 ±3.3	22.2 ±3.6	54.1 ±2.7	14.4 ±1.5	13.4 ±1.7	30.9 ±5.5
E. coli	48.6 ±2.3	45.5 ±1.5	84.6 ±2.7	18.9 ±1.8	17.2 ±0.9	47.6 ±3.5
P. putida	19.4 ±2.1	18.9 ±2.1	47.3 ±4.5	8.9±1.3	7.8±1.1	26.5 ±3.9

4.3.3. One-month stored freeze-dried cells toxicity assay

After 1-month of freeze-dried storage, PEG and sucrose/Tween 80 pre-treated freeze-dried bacterial strains stored at either 4 ˚C or -20 ˚C, responded to standard toxicants in a similar dose-dependent manner (data not shown) compared to the same cryoprotectant pre-treated zero-time stored freeze-dried counterparts (Figure. 4.2) but the EC_{50}'s were slightly lower (Table 4.2) than the same cryoprotectant pre-treated zero-time stored freeze-dried cells (Table 4.1). The differences, however, were all within the estimates of error.

EC_{50} of freeze-dried cells of each bacterial strain treated with the same cryoprotectant (either PEG or sucrose/Tween 80), stored at 4 ˚C was lower than the EC_{50} of cells stored at -20 ˚C. At the same storage temperature (either 4 ˚C or -20 ˚C), the EC_{50} of PEG pre-treated cells was lower than those cells pre-treated with sucrose/Tween 80 when exposed to standard toxicants (Table 4.2).

Table 4.2: EC_{50} of two cryoprotectants pre-treated freeze-dried cells after 1-month storage at two different temperatures (4 ˚C, -20 ˚C), when exposed to two standard toxicants (2,4-DCP, 3,5-DCP) (n = 3, P < 0.001).

Bacterial strains	EC_{50} of 1-month freeze-dried cells to standard toxicants (mg/l)							
	4 ˚C				-20 ˚C			
	PEG		Sucrose + Tween 80		PEG		Sucrose + Tween 80	
	2,4-DCP	3,5-DCP	2,4-DCP	3,5-DCP	2,4-DCP	3,5-DCP	2,4-DCP	3,5-DCP
A.calcoaceticus	19.0±2.4	11.6±1.7	37.1±2.2	25.5±1.3	21.5±2.1	12.5±1.4	41.5±3.8	29.6±3.0
E. coli	39.9±2.8	15.1±1.6	64.2±4.8	33.6±2.7	42.4±2.4	16.3±0.9	73.8±4.8	38.2±3.1
P. putida	17.2±1.2	6.7±0.8	33.3±2.6	18.0±1.5	17.8±1.9	7.1±0.9	36.6±3.5	24.8±2.4

4.3.4. Two-month stored freeze-dried cells toxicity assay

After freeze-drying and 2-month storage, all three freeze-dried strains stored at either 4 ˚C or -20 ˚C still responded to standard toxicants in a similar dose-dependent manner (data not shown), compared to the same cryoprotectant pre-treated, zero-time stored counterparts. Under same storage conditions (either 4 ˚C or -20 ˚C), the EC_{50} values of each bacterial strain to standard toxicants were lower (Table 4.3) after 2-month storage, compared with the same pre-treated 1-month stored freeze-dried counterparts (Table 4.2).

Similar to 1-month freeze-dried cells, the EC_{50} values of PEG pre-treated cells of 2-month stored bacterial strains were lower than those pre-treated with sucrose/Tween 80 and to the two standard toxicants under the same storage conditions (Table 4.3). Within the same cryoprotectant treatment, freeze-dried bacterial strains stored at 4 ˚C had lower EC_{50} values (on exposure to the two standard toxicants) than their -20 ˚C stored counterparts.

Table 4.3: EC_{50} of two cryoprotectants pre-treated freeze-dried cells after 2-month storage at two different temperatures, (4 ˚C, -20 ˚C), when exposed to two standard toxicants (2,4-DCP, 3,5-DCP) (n = 3, P < 0.001).

Bacterial strains	**EC_{50} of 2-month freeze-dried cells to standard toxicants (mg/l)**							
	4 ˚C				**-20 ˚C**			
	PEG		**Sucrose + Tween 80**		**PEG**		**Sucrose + Tween 80**	
	2,4-DCP	**3,5-DCP**	**2,4-DCP**	**3,5-DCP**	**2,4-DCP**	**3,5-DCP**	**2,4-DCP**	**3,5-DCP**
A.calcoaceticus	16.3±0.7	7.9±0.9	33.6±1.5	21.3±1.3	19.2±1.0	10.6±0.9	37.1±2.8	27.3±2.0
E. coli	32.8±2.5	11.9±1.3	53.4±3.5	29.7±2.3	37.9±1.2	13.6±1.7	60.9±2.5	32.3±2.5
P. putida	14.5±1.4	5.3±0.4	30.4±2.9	14.2±1.8	16.1±1.1	6.6±0.5	34.8±2.9	21.9±2.7

4.3.5. Three-month stored freeze-dried cells toxicity assay

After 3 months storage at 4 ˚C, two of the PEG pre-treated bacterial strains, *A. calcoaceticus* and *E. coli* did not respond to the two toxicants in the SciTox™ bioassay, whereas *P. putida* was more stable. Both these strains (4 ˚C stored PEG pre-treated *A. calcoaceticus* and *E. coli*) responded to standard toxicants in a random manner (data not shown). In contrast, the -20 ˚C stored PEG pre-treated counterparts of these two strains still responded to toxciants in a dose-dependent manner similar to their fresh counterparts. All three sucrose pre-treated freeze-dried strains (stored at either 4 ˚C or -20 ˚C) responded in a similar dose-response profile (data not shown) compared to their fresh cell counterparts. However, after 3-month storage, freeze-dried cells seemed to be more susceptible to toxicants as shown by lower EC_{50} values (Table 4.4), compared to their 1- and 2-month stored freeze-dried counterparts (Table 4.2 and 4.3).

Table 4.4: EC_{50} of two cryoprotectants pre-treated freeze-dried cells after 3-month storage at two different temperatures (4 ˚C, -20 ˚C), when exposed to two standard toxicants (2,4-DCP, 3,5-DCP) (n = 3, P < 0.001).

Bacterial strains	EC_{50} of 3-month freeze-dried cells to standard toxicants (mg/l)							
	4 ˚C				-20 ˚C			
	PEG		Sucrose + Tween 80		PEG		Sucrose + Tween 80	
	2,4-DCP	3,5-DCP	2,4-DCP	3,5-DCP	2,4-DCP	3,5-DCP	2,4-DCP	3,5-DCP
A.calcoaceticus	N/A	N/A	30.7±2.1	17.2±1.8	10.8±1.4	6.7±0.7	30.8±2.5	21.8±1.7
E. coli	N/A	N/A	50.5±1.8	26.8±2.0	30.3±2.4	10.8±1.1	51.4±4.1	27.5±2.8
P. putida	11.7±1.4	4.1±0.3	24.3±2.7	10.8±0.9	14.5±1.2	4.9±0.5	30.8±3.0	18.3±1.3

4.3.6. Cell viability study

In order to evaluate the effects of freeze-drying process and subsequent storage on cell viability among different factors (cryoprotectant and storage temperature), the cell viability of three freeze-dried strains based on plate count was studied at zero-time, 1-, 2- and 3-months, relative to their freshly cultured counterparts (Figure. 4.3). Within the same cryoprotectant pre-treatment freeze-dried cells, the cell viability of *P. putida* was highest among the three bacterial strains and *A. calcoaceticus* was the lowest after freeze-drying. In each bacterial strain, the cell viability of freeze-dried cells pre-treated with sucrose/Tween 80 was higher than those pre-treated with PEG.

After 1-month storage, there was a loss in cell viability in all three bacterial strains but the loss by *E. coli* (regardless of cryoprotectant) was greater than in the other two freeze-dried bacterial strains. *P. putida* still retained higher cell viability among the three freeze-dried bacterial strains. The loss in cell viability of freeze-dried bacterial strains treated with either PEG or sucrose/Tween 80 was more pronounced in the cells stored at 4 ˚C than at -20 ˚C. For each bacterial strain stored at the same temperature,

PEG pre-treated freeze-dried cells were less viable than the counterparts treated with sucrose/Tween 80.

After 2-month storage, PEG pre-treated *P. putida* still retained 38.5% and 45.1% of cell viability at 4 and -20 ˚C respectively. The corresponding values for *A. calcoaceticus* were 13.8% and 16.2% and *E. coli* fared the worst with values of 7.9% and 11.2%. The freeze-dried bacterial strains pre-treated with sucrose/Tween 80 were more viable than those treated with PEG. The cell viabilities of sucrose/Tween 80 pre-treated *P. putida* were the highest at 4 and -20 ˚C with values of 56.2 and 62.4% respectively. The corresponding values of *A. calcoaceticus* were 25.6 and 45.8% respectively similar to the *E. coli* values of 23.9 and 42.9%. Irrespective of the cryoprotectant, for each of three freeze-dried bacterial strains, cells stored at -20 ˚C were more viable than those stored at 4 ˚C.

After 3-month stored at 4 ˚C, the viability of PEG pre-treated *A. calcoaceticus* and *E. coli* were less than 5%, significantly lower than their -20 ˚C stored counterparts of 13.3% and 9% respectively (Figure 4.3). Similar to the previous 1- and 2-month storage results, sucrose/Tween 80 pre-treated freeze-dried cells showed significantly greater cell viability than PEG pre-treated counterparts (Figure 4.3). Regardless of the cryoprotectant or the storage temperature, freeze-dried *P. putida* had a higher cell viability compared to *A. calcoaceticus* and *E. coli*.

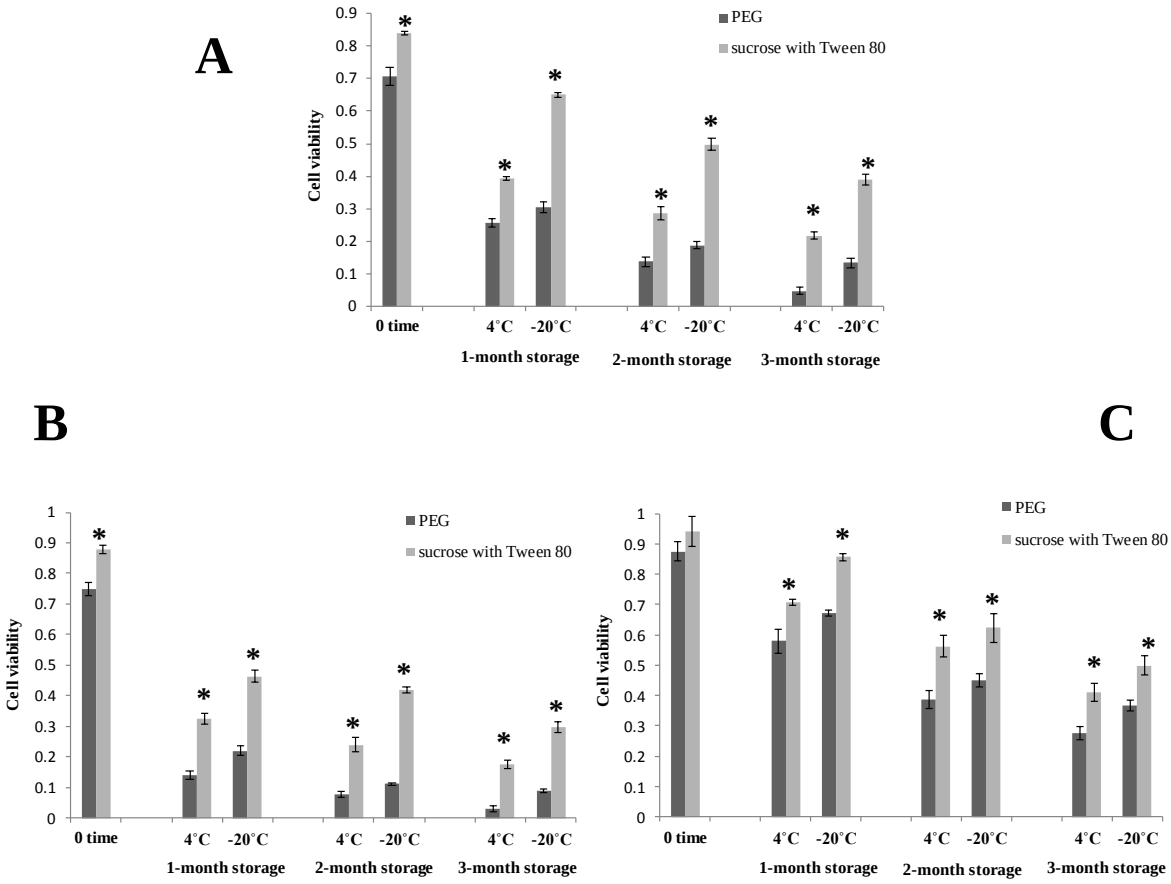

Figure 4.3. Cell viability of three freeze-dried bacterial strains pre-treated with two cryoprotectants after storage at four different time periods (0, 1, 2 and 3 months), at either 4 ˚C or -20 ˚C. Diagram A shows the cell viability of freeze-dried *A. calcoaceticus*. B *E. coli*, and C *P. putida*. *: significantly different from PEG pre-treated counterparts in a paired test at $P<0.01$. n = 3, error bars are standard error.

4.4. Discussion

Recently, freeze-dried cells have received increased attention for their use in environmental chemical toxicity assays (Choi and Gu 2002; Choi and Gu 2003; Gu et al. 2001; Shin et al. 2005). There are many factors that can affect the shelf life of freeze-dried cells (Champagne et al. 1996; Kuleshova et al. 1999; Kurtmann et al. 2009; Miyamoto-Shinohara et al. 2006). To our knowledge, the shelf life of freeze-dried cells used in toxicity assay has not been reported before. In this study, the shelf life of freeze-dried bacterial strains in SciTox^{TM} rapid DTA assay when exposed to

two standard toxicants (2,4-DCP and 3,5-DCP), was evaluated against three factors (bacterial strain, type of cryoprotectant and storage temperature) at three time periods (1, 2 and 3 months).

Of the three bacterial strains studied, *A. calcoaceticus*, *E. coli* and *P. putida*, (Tables 4.1-4.4), *E. coli* seemed to be the most resistant strain to both standard toxicants, whereas *P. putida* was the most sensitive strain. After freeze-drying (Figure. 4.2) and subsequent storage at either 4 or -20 ˚C for one and two months (data not shown), all three freeze-dried strains (pre-treated by either PEG or sucrose/Tween 80) responded to standard toxicants (2,4-DCP or 3,5-DCP) in a similar dose-dependent manner, compared to their freshly cultured counterparts (Figure. 4.1). However, the EC_{50} values to both standard toxicants became lower as storage time increased compared with the EC_{50} of their zero-time freeze-dried counterparts. This indicates that the freeze-dried cells were more sensitive to standard toxicants in SciTox^{TM} DTA assay following storage. Of the three freeze-dried bacterial strains, the EC_{50} of *E. coli* declined faster than the other two, during storage. After 3-month stored at 4 ˚C, both PEG pre-treated *A. calcoaceticus* and *E. coli* could not be used in the SciTox^{TM} assay, whereas *P. putida* remained relatively stable (Table 4.4).

Moreover, *A. calcoaceticus* seemed to be the most sensitive strain to freeze-drying, whereas *E. coli* was the most sensitive strain to subsequent storage after freeze-drying based on its cell viability loss rate (Figure. 4.3). In contrast, *P. putida* was the most resistant strain to both freeze-dry and subsequent storage after freeze-drying. However, *P. putida* has been reported to be more sensitive to freeze-drying and subsequent storage than *E. coli* (Miyamoto-Shinohara et al. 2006). In view of the results presented here, freeze-dried *P. putida* seemed to have the better shelf-life in SciTox^{TM} toxicity assay irrespective of the cryoprotectant and storage temperature compared to *A. calcoaceticus* and *E. coli*.

The exact reasons for the three bacterial strains having different rates of viability loss and the different sensitivities to toxicants following freeze-drying and subsequent storage, is not clear. Generally, the shelf life of freeze-dried cells can be affected by a multitude of factors. For example, the cell membrane could be damaged by freeze-drying process (Mazur 1970), resulting in increased membrane permeability (Sinskey and Silverman 1970). Additionally, dry cells from the frozen state could cause

additional decrease in cell viability. During freeze dried storage, oxidation plays a role causing a loss of cell viability (Castro et al. 1997).

The cryoprotectants play an important role in stability of freeze-dried cells. A cryoprotectant prevents formation of ice crystals and is commonly used to protect cells during freezing. However, it has been reported to cause cell damage, including osmotically mediated injury (Steponkus 1984) inhibiting the antioxidant activity of catalase and peroxidase (Gernaey et al. 1997).

In this study, all three freeze-dried PEG pre-treated bacterial strains seemed to be more susceptible to the two toxicants (2,4-DCP, 3,5-DCP) than their fresh cultured counterparts (Table 4.1). This is understandable because during freeze-drying and storage periods the cells sustain damage (e.g. membrane damage) and thus became more sensitive (e.g. increased permeability) to the toxicants. In contrast, the sucrose/Tween 80 pre-treated freeze-dried bacterial strains were less sensitive to toxicants (Table 4.1). The reason for this is not clear and was not pursued in this study. It is also interesting to note that the sucrose/Tween 80 pre-treated freeze-dried cells were even less sensitive to toxicants than their fresh counterparts. Clearly the presence of sucrose is providing some protection to the cells from the toxicants. It is also possible that the sugar acted as a nutrient for the bacteria and boosted their catabolism rate. For example, glucose has been commonly used as a substrate to improve cell respiratory activity in the SciTox^{TM} assay (Pasco et al. 2008). In a previous study, it was reported that the cell respiratory activity could be significantly increased by suspending the cells in sucrose-containing buffer solution (Hayek and Tipton 1966). Both PEG or sucrose/Tween 80 pre-treated freeze-dried cells seemed to be more susceptible to standard toxicants after storage, compared with their zero-time stored freeze-dried counterparts. However, the EC_{50} values of PEG pre-treated freeze-dried strains to the two standard toxicants in the SciTox^{TM} DTA assay seemed to be more reliable (more sensitive to toxicants than fresh counterparts) than sucrose/Tween 80 pre-treated freeze-dried counterparts.

Sucrose/Tween 80 pre-treated freeze-dried bacterial strains exhibited significantly higher cell viability after freeze-drying and subsequent storage than the PEG pre-treated counterparts (Figure. 4.3). The viability loss rate of PEG pre-treated freeze-dried cells during storage, was much greater than the sucrose/Tween 80 pre-treated

counterparts. A similar result to this has been reported by others (Gayle et al. 2006; Kuleshova et al. 1999). Freeze-dried cells that were pre-suspended in sugar mixture (sucrose with glucose) solution exhibit better cell viability than those pre-suspended in PEG-containing solutions (Gayle et al. 2006). It also has been reported that sucrose pre-treated freeze-dried cells had greater cell viability than both glucose and glucose with sucrose pre-treated freeze-dried cells (Shin et al. 2005). Additionally, the viability of freeze-dried cells could be further improved by combined sucrose with Tween 80 (Choi and Gu 2002). The reason that sucrose/Tween 80 pre-treated freeze-dried cells had better cell viability than PEG, might be due to their different toxicities. Sucrose has been reported to have low toxicity compared with PEG (Kuleshova et al. 1999). Additionally, Tween 80 that was used in combination with sucrose is considered to have little or no anti-bacterial activity (Dawson et al. 1986).

It is known that the shelf life of freeze-dried cells can be significantly improved by lowering the storage temperature (e.g. -70 ˚C) (Shin et al. 2005) and by applying a vacuum (Miyamoto-Shinohara et al. 2006). However, for easy storage and to facilitate handling of freeze-dried cells when performing frequent commercial toxicity tests (e.g. SciTox^{TM}), two readily accessible storage temperatures 4 ˚C (refrigerator temperature) and -20 ˚C (freezer temperature) were selected under normal laboratory conditions (at normal atmospheric pressure) for this study. All three freeze-dried bacterial strains responded to standard toxicants in a similar dose-dependent manner after 1- and 2-month storage at either 4 or -20 ˚C (data not shown), compared with their fresh (Figure. 4.1) and zero-time stored freeze-dried counterparts (Figure. 4.2). However, the freeze-dried cells stored at 4 ˚C seemed more susceptible (lower EC_{50}) to standard toxicants than those stored at -20 ˚C. After 3-month storeage at -20 ˚C, all three freeze-dried bacterial strains responded to standard toxicants in dose-dependent manners and acted similar to the fresh cells irrespective of the cryoprotectant used. In contrast, 4 ˚C stored PEG pre-treated *A. calcoaceticus* and *E. coli* could not used to assay toxicity of 2,4-DCP and 3,5-DCP in SciTox^{TM} assay, due to a heavy loss of their cell viability. The viability loss rate varies with the storage temperature (Champagne et al. 1996). The rate of viability loss in 4˚C stored freeze-dried strains was faster than their -20 ˚C stored counterparts (Figure. 4.3). This is similar to the result of a previous study (Champagne et al. 1996). Based on this, freeze-dried strains

offer more reliable EC_{50} values (closer to EC_{50} obtained by fresh counterparts) probably because of the higher cell viability when stored at -20 ˚C than at 4 ˚C.

4.5. Conclusions

The shelf-life of freeze-dried cells in SciTox™ DTA assay was studied in this research. *P. putida* was the most resistant strain to freeze-drying and subsequent storage, and more stable in toxicity assay than *A. calcoaceticus* and *E. coli*. Cell viability was significantly improved by using sucrose/Tween 80 as a cryoprotectant compared to PEG which is the more preferred one in SciTox™ toxicity assay and hence produced more reliable reproducible EC_{50} values. Cells stored at -20 ˚C exhibited a greater viability and hence a longer shelf-life compared to their 4 ˚C stored freeze-dried counterparts and hence gave good results in the SciTox™ assay. It is concluded that all three freeze-dried bacterial strains pre-treated with PEG could be stored at -20 ˚C for 3-months and still be used in the SciTox™ DTA assay.

CHAPTER 5: DEVELOPMENT OF GENETICALLY MODIFIED SCTIOX™ BIOSENSORS

5.1 Introduction

Genetically engineered microorganisms utilizing reporter genes fused to inducible promoters have been applied to assay toxicity. These whole-cell biosensors only produce a response to a specific substrate (Chinalia et al. 2008; del Busto-Ramos et al. 2008; Lehmann et al. 2000; Tag et al. 2007). Typically, these biosensors have utilized visual reporter genes such as *gfp* (Fiorentino et al. 2009), *lux* (Chinalia et al. 2008) and *lacZ* (Freire-Picos and Lamas-Maceiras 2006). However, some of them, similar to SciTox™, are developed based on amperometric measurement.

The *lacZ* gene has been used as a reporter gene in an amperometric biosensor to detect Cu in yeast (Lehmann et al. 2000; Tag et al. 2007). In this biosensor, *lacZ* was fused to the Cu-inducible *CUPI* promoter. Thus the constructed yeast biosensor responded to the presence of Cu by synthesising β-galactosidase, a product of *lacZ* gene. The enzyme, β-galactosidase, then cleaved the additional substrate lactose to glucose and galactose. The catabolism of these substrates causes a decrease in oxygen concentration, which is measured amperometrically by an oxygen electrode.

In this study we have attempted to use substrate-specific gene induction to directly induce expression of a respiration gene, thus directly linking respiration rate to the presence of the specific substrate. In order to create a bio-assay that could specifically identify and quantify antibiotics, the SciTox™ assay bio-component was re-engineered to contain a reporter gene and an inducible promoter fusion system. To do this, the research was divided into three parts.

In the first part we searched for reporter genes suitable for use in the SciTox™ amperometric biosensor. To achieve this, *E. coli* single-gene mutants from the Keio collection (Baba et al. 2006) were screened in SciTox™ assay to find out genes essential for the SciTox™ response. In our laboratory, the entire Keio collection (Baba et al. 2006) of approximately 4000 single gene knock-out *E. coli* strains has

been screened for genes putatively involved in the SciTox^{TM} response (Weld et al. 2010). Of the 4000 genes tested, over 100 were identified as potentially contributing to a higher SciTox^{TM} signal. These genes were involved in a number of different cellular processes including transmembrane transport, stress response, respiration, and fermentation. Initially, knock-out genes from strains that gave the lowest SciTox^{TM} signals were selected to confirm their role in SciTox^{TM} response. From these, three genes were selected for potential use in development of the amperometric biosensor strain on the basis of their different cellular pathways. These genes were: *selA*, *nuoA* and *ompF*.

The *selA* gene encodes for the enzyme selenocysteine synthase (Tormay et al. 1998b), which plays an important role in the reaction of serine to selenocysteine conversion on Ser-tRNA^{Sec} by using monoselenophosphate as the selenium (Se) donor (Allmang et al. 2009; Stock et al. 2010). The *selA* gene belongs to the *sel* operon which encodes for selenocysteine (Allmang et al. 2009). Selenium atoms have similar properties to sulphur (S) atoms. However, by using Se instead of S, the catalytic rate of seleno-proteins is significantly increased (Tormay et al. 1998a). Therefore, by manipulating the expression of *selA* it should be possible to manipulate the overall redox activity of the respiratory enzymes.

The outer membrane protein F (ompF) encoded by the *ompF* gene, forms a large porin channel involved in passive diffusion across the outer membrane of Gram-negative bacteria, including *E. coli* (Bekhit et al. 2011; Garcia-Gimenez et al. 2011; Housden et al. 2010). The entry of antibiotics into bacterial cells is mainly regulated by the function of the ompF protein (Housden et al. 2010; Sambrook and Russell 2001). Therefore, up-regulation of ompF expression might enhance antibiotics (e.g. Tet) and SciTox^{TM} mediator (e.g. KFCIII) entry efficiency through the cell membrane.

The *nuo* operon contains 13 genes *nuoA-N*, which encodes for subunits of NADH dehydrogenase I. NADH dehydrogenase I plays an important role in the *E. coli* respiratory chain (Zhang et al. 2004). NADH dehydrogenase I in *E. coli* catalyses the electron transfer reaction from NADH to ubiquinone (the first step in *E. coli* ETC) (Calhoun et al. 1993). By inducing *nuoA* gene expression, respiratory activity could be up-regulated.

In the second part, an antibiotic-inducible promoter was selected and its functioning and response to the target analyte was characterised. As an inducible promoter, the *tetA* promoter was chosen from the well characterized *E. coli* transposon *Tn10* and the target analyte used was Tet. The *Tn10 Tet* operon encodes proteins which confer Tet resistance in response to the presence of Tet (Aleksandrov et al. 2008). The *Tn10 tetA* promoter can be up-regulated by the antibiotic Tet and/or its epimers (Virolainen et al. 2008).

Neither the reporter genes nor the *tetA* promoter have previously been used as an inducible genetic reporter system in the SciTox™ assay. Prior to combining the two components in one construct, the *tetA* promoter was fused to the visual reporter gene *lacZ*. This allowed the promoter response to different concentrations of Tet to be tested. This construct also allowed determination of appropriate assay conditions for gene induction and expression.

In the third part, a genetically modified whole cell biosensor was constructed by fusing an inducible promoter to a reporter gene which could be used to detect and quantify a specific analyte in the SciTox™ system. To do this, four different strategies were designed to construct specific SciTox™ biosensors, all based on gene induction in response to a specific substrate.

In the first strategy, the quantity of metabolizable carbon available to the bacteria in the SciTox™ assay was manipulated. The metabolizable carbon source (glucose) used to increase SciTox™ signal was only available in the presence of Tet. This was achieved by fusing the inducible *tetA* promoter to the *lacZ* gene, with *lacZ* expression induced by Tet resulting in conversion of metabolically unavailable lactose to the metabolizable substrate glucose and galactose (Lehmann et al. 2000; Tag et al. 2007).

In the second strategy, the overall redox activity of respiratory enzymes was manipulated. For this method, the *selA* gene, which encodes the enzyme selenocysteine synthase, was fused to *tetA* promoter and transferred into the *selA* knock-out (Δ*selA*) *E. coli* strain. Therefore, the expression of *selA* was regulated by Tet.

In the third strategy, the passive diffusion efficiency of a mediator (KFCIII) across the out membrane of *E. coli* sensor cells was manipulated. For this method, *ompF* gene

was fused to *tetA* promoter and transferred into the *ompF* knock-out (Δ*ompF*) *E. coli* mutant. Thus the SciTox™ related activity might be increased due to the improved entry efficiency of KFCIII through cell membrane in the presence of Tet.

In the fourth strategy, the key respiration gene *nuoA* was placed under the control of the *tetA* promoter, so that in the presence of Tet, the gene was expressed and the cellular respiration rate increased. For this method, *nuoA* was fused to *tetA* promoter and then transferred into the *nuoA* knock-out (Δ*nuoA*) strain.

Of the four strategies, *nuoA* based assay exhibited a highly specific and sensitive response to Tet. To confirm the *nuoA* reporter gene based SciTox™ amperometric biosensor could be used to detect environmental compounds other than Tet, the *nuoA* expression construct was re-engineered, placing the *nuoA* gene under the control of a different inducible promoter. For this work, the *copA* promoter, which is induced by Cu^{2+} and Ag^{+} ions, was used.

The *copA* promoter regulates the expression of the *copA* gene (Stoyanov and Brown 2003), which encodes a Cu-translocating P-type ATPase, known as CopA protein (Rensing et al. 2000). The CopA protein plays a role in Cu export and resistance (Stoyanov and Brown 2003). The *copA* promoter is controlled by protein CueR (Stoyanov et al. 2001). The CueR protein has been reported to activate the *copA* promoter in the presence of Cu (Rensing et al. 2000) and Ag (Stoyanov et al. 2001) (Stoyanov and Brown 2003). To construct Cu/Ag specific biosensor, the *copA* promoter was fused to *nuoA* reporter gene in plasmid pSong11 and then transferred into the Δ*nuoA* strain. Therefore, the expression of *nuoA* was driven by the *copA* promoter in response to the concentrations of Cu^{2+}/Ag^{+}. In order to confirm the specificity and sensitivity of Δ*nuoA* pSong11 bioassay, both unmodified and non-specific assays were manipulated.

5.2 Methods

Construction of pSong8: The Multi-cloning site (MCS) of pBluescript was replaced with a SalI restriction enzyme site by PCR using primers 5'-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3' and 5'- ATGA **GTCGAC** CAA TTC GCC CTA

TAG TGA GT-3' followed by SalI digestion and re-ligation. The *Lac* promoter region upstream of *lacZ* gene was removed by PCR using primers 5'-ATAG **CTGCAG** ACT GCC CGC TTT CCA GTC GG-3' and 5'- AGGAA **AAGCTT** ATG ACC ATG ATT ACG CCA AGC. The product was digested with *PstI* and *HindIII* and ligated to the Tn10 *TetA* promoter amplified by PCR from *E. coli* genomic DNA using primers 5'-ATCAC **CTGCAG** AAT GGG AAT TGA CGT TCC TTC-3' and 5'- AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3'.

Construction of pSong9 (P_{tetA} fused with the *selA* gene): The *lacZ* gene region was removed from (pSong8) by PCR with primers 5'- AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3' and 5'-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3'. The PCR product obtained was (pSong8) sequence lacking the *lacZ* gene and this product was digested with *HindIII* and *SalI* restriction enzymes and ligated to the *E. coli selA* gene amplified from *E. coli* genomic DNA by PCR using primers 5'- ATCA **AAGCTT** ATG ACA ACC GAA ACG CGT TC-3' and 5'- ATAG **GTCGAC** CAG AGA TAT CGC GCA ATA CC-3' also digested with *HindIII* and *SalI* restriction enzymes.

Construction of pSimon (P_{tetA} fused with *ompF* gene): The *lacZ* gene region was removed from (pSong8) by PCR with primers 5'- AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3' and 5'-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3'. The PCR product obtained was (pSong8) a sequence lacking the *lacZ* gene and this product was digested with *HindIII* and *SalI* restriction enzymes and ligated to the *E. coli ompF* gene amplified from *E. coli* genomic DNA by PCR using primers 5'- ATCA **AAGCTT** CAT GAG GGT AAT AAA TAA TGA TG -3' and 5'- ATAG **GTCGAC** TCG GCA TTT AAC AAA GAG GTG -3' also digested with *HindIII* and *SalI* restriction enzymes.

Construction of pSong10 (P_{tetA} fused with the *nuoA* gene): The *lacZ* gene region was removed from (pSong8) by PCR with primers 5'- AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3' and 5'-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3' as described above. The PCR product was digested by *HindIII* and *SalI* restriction enzymes and ligated to the *E. coli nuoA* gene amplified from *E. coli* genomic DNA by PCR using primers 5'- ATCA **AAGCTT** ATG AGT ATG TCA

ACA TCC ACT-3' and 5'- ATAG **GTCGAC** TCA ACC GGG ATG AAT TTA TCG-3' also digested with *HindIII* and *SalI* restriction enzymes.

Construction of pSong11 (P_{copA} fused with the *nuoA* gene): The *tetA* promoter was removed from (pSong10) by PCR with primers 5'- AATCA **AAGCTT** TT TTC TCT ATC ACT GAT AGG GA-3' and 5'-ATAG **GTCGAC** CAG CTT TTG TTC CCT TTA GTG-3'. The PCR product was digested by *HindIII* and *SalI* restriction enzymes and ligated to the *copA* promoter amplified from *E. coli* genomic DNA by PCR using primers 5'-ATCAC **CTGCAG** ATT TTG TCC GCC GTT AAG TG-3' and 5'-TGAC **AAGCTT** CAC TCC TTT AAG ACA GTT TTG ACT-3' also digested with *HindIII* and *SalI* restriction enzyme.

Cell culture: All cells were cultured in a shaking flask culture for 12 hours, in LB medium at 200 rpm, 37 ℃, and supplemented where appropriate with Amp 100 μg ml^{-1} and Kan 50 μg ml^{-1}.

Blue/white colour screen using pSong8

LB Petri-dish solid assay: Top10f' derivatives, with or without the specific plasmids, were grown at 37 ℃ for 16-20 h on LB agar plates, supplemented with ampicillin, Tet and 0.5 mM IPTG and 20 μg ml^{-1} X-gal.

96-well plate liquid assay: Top10f' derivatives, with or without the specific plasmids, from freshly grown overnight cultures (including three different growth phases: late-exponential phase, early-stationary phase and late-stationary phase) were harvested by centrifugation at 13K rcf at room temperature. The cells were washed three times in phosphate buffer (KH_2PO_4 10.62 mg L^{-1}, K_2HPO_4 21.25 mg L^{-1}, KCl 7.46 mg L^{-1}). The cell concentration was adjusted to an optical density at 600 nm (OD_{600}) of 5, measured using an UNICAM 8625 UV/VIS spectrophotometer. The assay was performed in 24-well microtest plates**.** 925 μl of cells were incubated with 1 μl of variable concentrations of Tet at 37 ℃, 200 rpm for six different periods (0, 15, 30, 60, 90mins and 16 hours), and then 75 μl of 252 mM lactose was added and incubated 37 ℃, 200 rpm. The time for blue colour development was monitored by using UNICAM 8625 UV/VIS spectrophotometer.

SciTox™ assay

Two different methods were used for preparing cells for the SciTox™ assay. The method chosen for each strain was the method that worked best for that strain in initial experiments.

Stationary-phase cell assay: *E. coli* from freshly grown overnight cultures were harvested by centrifugation at 13K rcf at room temperature. The cells were washed three times in phosphate buffer (KH_2PO_4 10.62 mg L^{-1}, K_2HPO_4 21.25 mg L^{-1}, KCl 7.46 mg L^{-1}). The cell concentration was adjusted to an optical density at 600 nm (OD_{600}) of 5, measured using an UNICAM 8625 UV/VIS spectrophotometer. The SciTox™ assay was performed in 24-well microtest plates. 1 ml of cells, were incubated with 1 µl of variable concentrations of Tet at 37°C, 200 rpm for 2 h; then 300 µl of 250 mM KFCIII was added to each well.

Exponential-phase cell assay: *E. coli* was grown until late-exponential growth phase (OD_{600} was around 0.5-0.8). The cells were not harvested by centrifugation but maintained in culture media. The SciTox™ assay was performed in 24-well microtest plates. 1 ml of cultured cells was incubated with 1 µl of variable concentrations of analytes (Tet/Cu^{2+}/Ag^{+}). The plates were incubated at 37 ℃, 200 rpm for 2 h; then 300 µl of 250 mM KFCIII was added to each well.

For both methods, the microtest plates were wrapped in aluminium foil to exclude light, and incubated in an orbital shaker at 37 ℃, 200 rpm for 1 h. Assays were terminated by transferring the contents of each well into 1 ml Eppendorf tube and centrifuged at 13K rcf for 4 min to pellet cells. The supernatants were transferred into clean Eppendorf tubes. The samples were stored at 4 ℃ until microelectrode amperometry analysis could be performed.

Calculation of Limit of Detection

Detection limit = 3.3 σ / S (Group 2005)

Where σ = the residual standard deviation of a regression line over the linear range

S = the slope of the calibration curve

5.3 Results

5.3.1 Candidate reporter genes from *E. coli*

In order to find suitable reporter genes for use in the amperometric SciTox™ biosensor, three genes were selected out of 100 genes that had previously been identified from a rapid screen of the 4000 strains of the Keio collection. The rapid screen had indicated that each of the 100 genes was putatively involved in the SciTox™ response. The three selected genes were *selA*, *ompF* and *nuoA*. They were selected on the basis that loss of each gene contributed to a significant reduction of SciTox™ response. Also, we selected genes that had significantly different modes of action to improve the chances of finding an optimal reporter gene. In order to confirm their role in the SciTox™ assay, strains with deletions of each of the genes were compared in the SciTox™ assay (Figure 5.1).

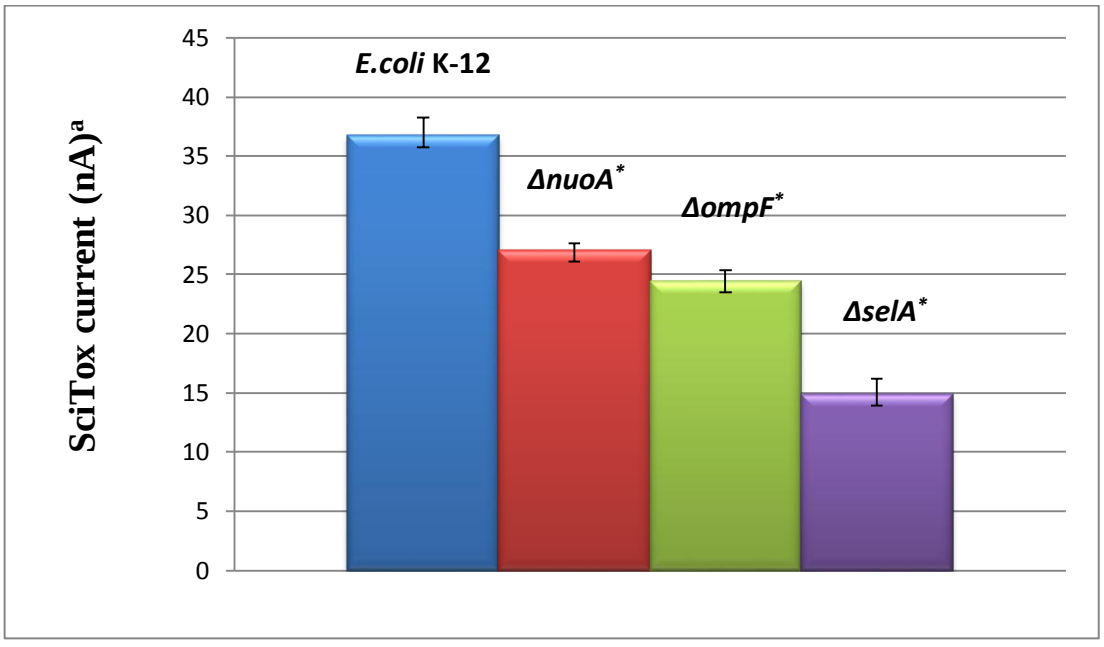

Figure 5.1. Respiratory activity comparison between non-mutated and three single-gene knock-out *E. coli* strains in SciTox™ assay. The name of mutated strains is given above the corresponding data bar. *: significantly different from the non-mutated in a paired test at $P < 0.01$. n=4, error bars are standard error.

The results showed that the SciTox^{TM} signal was significantly reduced by the knock-out of each of the three genes, compared with non-mutated *E. coli* strain. Therefore, we decided that genes *nuoA*, *ompF*, and *selA* were suitable candidates for amperometric reporter genes in the SciTox^{TM} assay.

5.3.2 Test Tet-inducible promoter (*tetA*) by using *lacZ* reporter gene

The *Tn10 tetA* promoter was chosen as our inducible promoter for antibiotic detection because it is a well characterized promoter that is strongly repressed and is specifically induced in low concentrations of Tet (Flache et al. 1992; Gatz and Quail 1988). In order to confirm that the *Tn10 tetA* promoter could be induced specifically by Tet, the promoter was inserted into plasmid pBluescript in place of the *lac* promoter, so that the expression of the *lacZ* reporter gene was controlled by the *tetA* promoter (*PtetA::lacZ*). Induction of *lacZ* expression by Tet was then tested in a blue-white colour assay. The colonies that carry the transgene (*PtetA::lacZ*) showed dark-blue colour in the presence of Tet in contrast to white-colour colonies of *E. coli* strains without the transgene. The assay confirmed the function of *Tn10 tetA* promoter.

The cell growth phase might play a role in the efficiency of reporter gene expression. To determine the optimal growth-phase for Tet induction, cells used in the assay were harvested at three different growth phases: exponential-, early stationary- and late stationary phases (Table 5.1). The optimal growth phase was determined by comparing the blue colour formation rate between cells used in different growth phases. In the presence of Tet, late-stationary phase cells exhibited the most rapid rate of blue-colour development. However, cells in this stage also developed blue-colour in a short time (80 mins), even without Tet. In contrast, early-stationary phase cells contributed to a moderate blue-colour formation rate in the presence of Tet and no observed blue-colour within 200 mins in the absence of Tet. However, the lowest blue-colour formation rate was acquired by exponential-growth phase cells. Therefore early stationary-phase cells were used for Top10f' pSong8 assay. Once the optimal growth-phase was determined, the optimal Tet-inducible period could be resolved.

Table 5.1. Comparison of blue color development with 3 different growth phases

	Con. of Tet ($5\ \mu g\ ml^{-1}$)	Growth phase (hour)		
		8	**12**	**16**
Time of blue-colour formation (mins)	**Induced by Tet for 30 mins**	200	80	50
	Negative control (no Tet)	Not observed	200	80

In order to optimize Tet-induction, Top10f'pSong8 biosensor (early-stationary phase cell) was induced by Tet at six different periods (0, 15, 30, 60 and 90 mins and 16 hours) and then incubated with X-gal for 180 mins. In general, the time used for blue-colour development by Top10f'pSong8 reduced as the Tet induction period increased. However, at Tet induction times greater than 90 mins, there was only a slight effect on blue-colour formation rate. Therefore, 90 mins was used for *tetA* promoter induction. Based on above findings, *Tn10 tetA* promoter could be used for construction of Tet-specific biosensors by fusing it with separate reporter genes (*lacZ*, *selA*, *ompF* and *nuoA*) in SciTox^{TM} assay.

5.3.3 Response of unmodified *E. coli* assays to Tet

In order confirm that the specificity and sensitivity of genetically modified SciTox^{TM} assay could be improved by induction in response to a specific analyte (Tet), the unmodified *E. coli* K12 BW25113 (does not have genetically constructed plasmids) sensor was used as a negative control and its response to a broad range of concentrations of Tet was tested (Figure 5.2). For this assay, cells that harvested at both stationary and exponential phases were preformed, thereby finding an optimal growth phase use for genetically modified SciTox^{TM} bioassay.

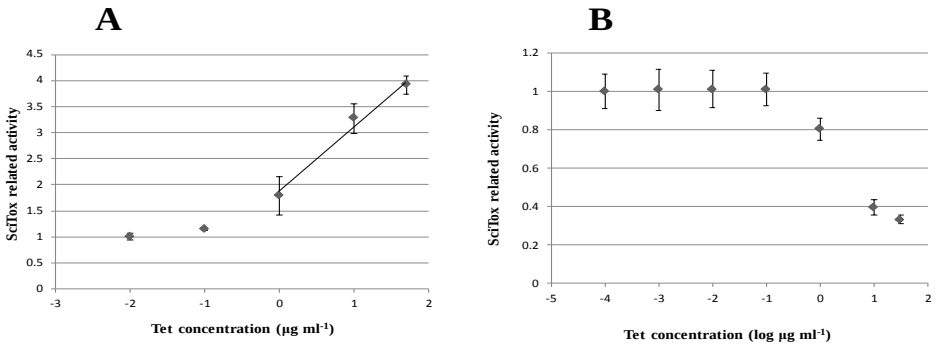

Figure 5.2. SciTox^{TM} response (Mean + SD) of *E. coli* K12 BW25113, exposed to a range of Tet concentrations in water samples. A: Response of stationary phase cells; the fitted linear regression line is y = 1.27x + 1.85, R^2 = 0.98. B: Response of exponential phase cells.

The unmodified *E. coli* strain did respond positively to Tet at sub-inhibitory levels with a DL of 4.11 µg ml^{-1} (Figure 5.2 A). The assay indicated that the unmodified *E. coli* assay responded to Tet at sub-inhibitory level. The reason why unmodified early-stationary phase *E. coli* assay responded to Tet in sub-inhibitory levels is not clear. Stationary phase cells were used as these were effective and convenient for use in the SciTox^{TM} assay. Also, previous comparison between early-stationary phase and exponential phase cells in *lacZ*-based blue-white colour assay (Table 5.1) indicated that stationary phase cells gave a more sensitive response than exponential phase cells. However, stationary phase cells seemed not suitable for use in genetically modified SciTox^{TM} bioassays due to the unexplained dose-dependent manner to Tet at sub-inhibitory levels.

However, by using exponential phase cells, there was no dose-dependent response detected at concentrations of Tet below sub-inhibitory levels (Figure 5.2 B). The loss of signal at high concentrations of Tet was due to the toxicity of Tet. Above results indicated that unmodified *E. coli* assay did not responded specifically and sensitively

to Tet by using exponential phase cells. Based on this, the exponential phase cells were used in all of the following genetically modified *E. coli* assays.

5.3.4 Assay based on *lacZ* expression

The enzyme β-galactosidase (products of *lacZ*) can cleave lactose into galactose and glucose. The latter product has been used as a substrate to enhance cell respiratory activity in the SciTox™ toxicity assay. In the presence of lactose, Tet could be detected through an increased SciTox™ signal. However, the Top10f' pSong8 assay did not respond to Tet in a dose-dependent manner. This is difficult to explain because we know that β-galactosidase expression was induced by Tet. The synthesis rate of β-galactosidase was relatively slow (Table 5.1) during the exponential growth phase. The low yield of glucose might have been insufficient to produce a detectable change in respiration during the SciTox™ assay.

5.3.5 Assay based on *selA* expression

In *ΔselA* pSong9 assay, the expression of the *selA* gene, encoding selenocysteine synthase, is induced in response to Tet. Expression of *selA* increases the activity of seleno-proteins including some proteins with redox activity. However, *ΔselA* pSong9 assay showed a similar dose-dependent manner to Tet as the negative control, unmodified *E. coli* assay (Figure 5.2 B). It is not known why the assay did not work. It has been shown that knockout of *selA* gene could significantly reduce cell respiratory activity (Weld et al. 2010). It is not directly the action of SelA protein but the action of the newly synthesized seleno-proteins after the induction of *selA* that increase respiration rate. The turn-over and expression of seleno-proteins may be quite low during the SciTox™ assay, and the respiration rate of sensor cells might not be affected by low levels of seleno-proteins. While the knock-out strain may have impaired function, induced expression from a complementing gene may not have been sufficient to restore function within the timeframe of this experiment.

5.3.6 Assay based on *ompF* gene expression

The *ompF* gene encodes protein ompF which plays an important role in passive diffusion of small molecules into and out of the periplasm of Gram-negative bacteria. By using *ompF* as a reporter gene, the SciTox^{TM} signal could be increased by improving the entry efficiency of the mediator (FKCIII), in the presence of Tet.

5.3.6.1 Detection of Tet in water sample by *ompF*-based SciTox^{TM} assay

The response of *ΔompF* pSimon assay to a broad range of concentrations of Tet in water samples was tested in the SciTox^{TM} assay. The *ΔompF* pSimon assay showed a sensitive dose-dependent response to Tet (Figure 5.3). The limit of detection and the linear dose-response range of Tet determined by *ΔompF* pSimon assay was 0.013 μg ml^{-1} and 0.01 – 0.1 μg ml^{-1} (Figure 5.3 B), respectively.

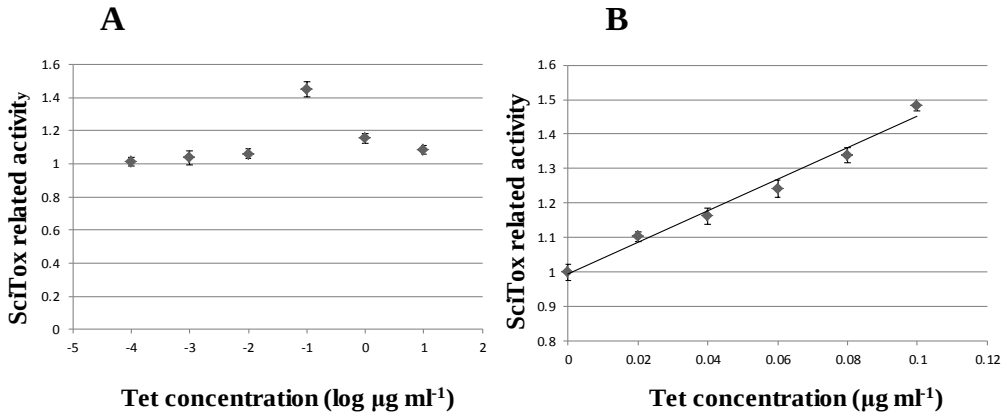

Figure 5.3. SciTox^{TM} response (Mean + SD) of *ΔompF* pSimon exposed to Tet in water sample. A: broad concentration range. B: dose-dependent range. The fitted linear regression line is y = 4.57x +0.99. R^2 = 0.98 for the dose-dependent data range.

5.3.6.2 Non-specific *ompF* assay response to Amp and Kan

In order to confirm the specificity of *ΔompF* pSimon assay to Tet, the sensor strain was tested at a broad range of concentrations of Amp and Kan. The Δ*ompF* pSimon assay did not respond to sub-inhibitory level of Amp and Kan in dose-dependent manners (Figure 5.4). These results confirmed that the response of Δ*ompF* pSimon to Tet (Figure 5.3) is not a general response to any other inhibitory antibiotics (e.g. Kan, Amp).

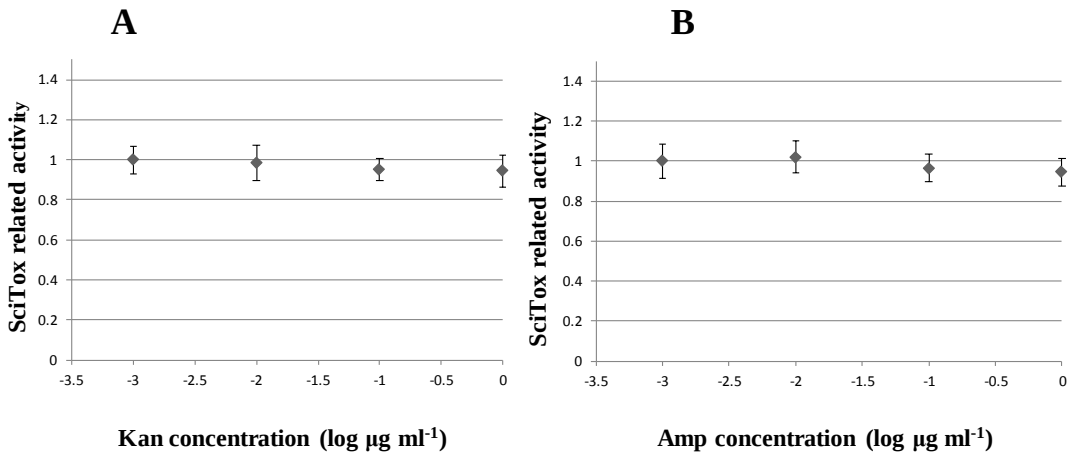

Figure 5.4. SciTox^{TM} response (Mean + SD) of Δ*ompF* pSimon sensor, exposed to Kan (A) and Amp (B) concentrations in water sample. There was no dose-dependent response observed below sub-inhibitory levels for both assays.

5.3.7 Assay based on *nuoA* gene expression

The *nuoA* gene is a key respiratory gene in the *nuo* operon, which encodes for NADH dehydrogenase I, an enzyme that plays an important role in the bacterial electron

transport chain. By using *nuoA* as a respiratory reporter gene, cell respiration rate was directly linked to induction of the gene.

The response of *ΔnuoA* pSong10 assay to a broad range of concentrations of Tet in water samples was tested in the SciTox^{TM} assay (Figure 5.5). The Δ*nuoA* pSong10 assay responded to Tet in a dose-dependent manner. The entire dose-response relationship could be described as: at very low concentrations of Tet (10^{-5} – 10^{-3} μg ml^{-1}), there was no dose-dependent response detected; at higher Tet concentrations (0.001-0.1 μg ml^{-1}), there was a significant increase in the SciTox^{TM} signal; then the SciTox^{TM} signal declined when Tet was above 0.1 μg ml^{-1} might due to the toxicity of Tet (Figure. 5.5 A). The DL was 0.0026 μg ml^{-1} (Figure. 5.5), which is about one order of magnitude more sensitive than the DL of *ΔselA* pSimon assay (Figure 5.3). The linear dose-response range to Tet was from 0 to 0.01 μg ml^{-1} (Figure. 5.5 B). This result indicated that the sensitivity of SciTox^{TM} assay could be improved by using the respiratory reporter gene (*nuoA*). Additionally, the Δ*nuoA* pSong10 assay did not respond to Amp and Kan at sub-inhibitory levels, confirming the specificity of the Δ*nuoA* pSong10 assay.

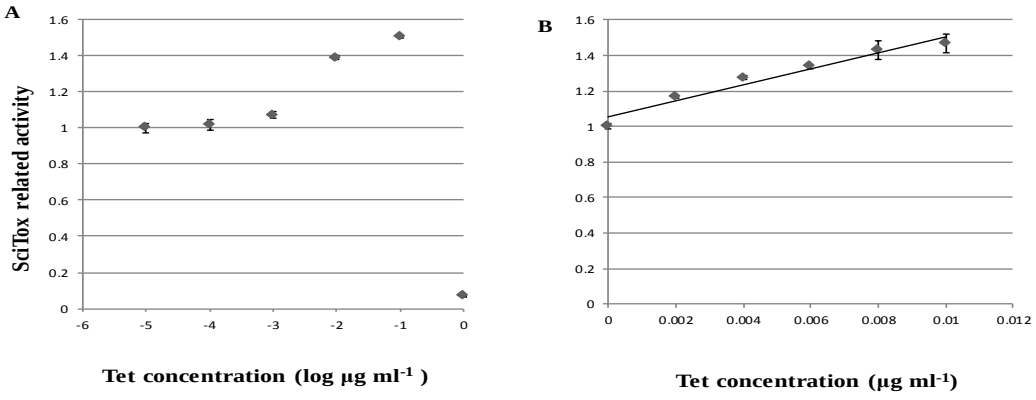

Figure 5.5. SciTox^{TM} response (Mean + SD) of sensor Δ*nuoA* pSong10, exposed to different Tet concentrations in water. A: broad concentration range. B: dose-dependent range. The fitted linear regression line is y = 45.75x + 1.05, R^2 = 0.96 for the dose-dependent data range.

5.3.8 The *nuoA*-based metal ions detection assay

In order to confirm that the inducible SciTox™ biosensor would be capable of detecting other substrates by using different inducible promoters, the *tetA* promoter was replaced with the *copA* promoter. The *copA* promoter is known to be controlled by protein CueR (Stoyanov et al. 2001), which has been reported to activate the *copA* promoter in the presence of Cu (Rensing et al. 2000) and Ag (Stoyanov et al. 2001) (Stoyanov and Brown 2003). The *nuoA* gene was fused to the *copA* promoter. Thus the expression of *nuoA* gene could be regulated by the presence of Cu^{2+} and/or Ag^{+} concentrations.

5.3.8.1 Detection of Cu^{2+} and Ag^{+} in water sample by using *nuoA*-based SciTox™ bioassay

To confirm that the *nuoA*-based bioassay could detect Cu^{2+} and Ag^{+} amperometrically, the response of Δ*nuoA* pSong11 was tested at a board range of $CuSO_4$ and $AgNO_3$ concentrations (Figure 5.6).

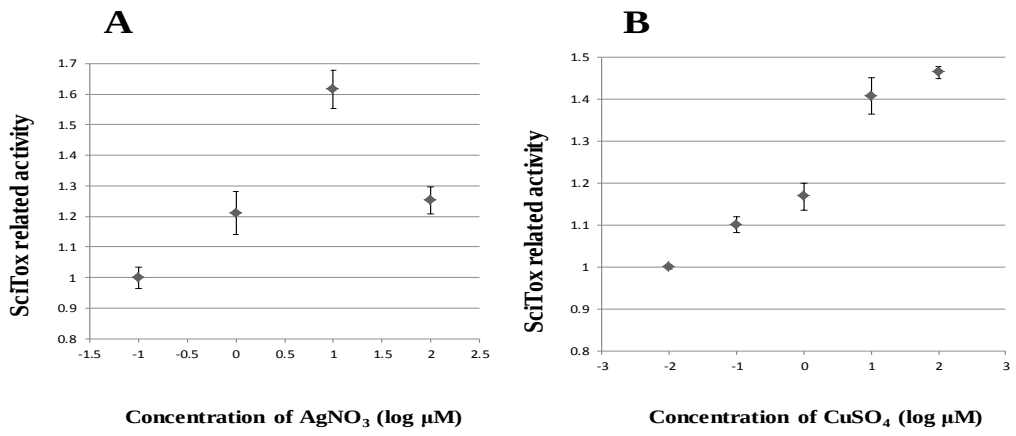

Figure 5.6. SciTox™ response (Mean + SD) of Δ*nuoA* pSong11 sensor, exposed to $AgNO_3$ (A) and $CuSO_4$ (B) concentrations in water sample. Both assays showed a dose-dependent manner.

The Δ*nuoA* pSong11 strain responded to Cu^{2+} and Ag^{+} in separate dose-dependent manners (Figure 5.6). The assay responded to Ag^{+} in a "mountain-shaped" dose-response (Figure 5.6 A), and to Cu^{2+} in a quantitative response within the given concentrations (Figure 5.6 B). These results indicated that *nuoA*-based SciTox™ bioassay could be used to detect heavy metals, Ag^{+} and Cu^{2+} at μM levels.

5.3.8.2 The Δ*nuoA* pSong11 specificity test

In order to confirm the specificity of the Δ*nuoA* pSong11 bioassay, a specific assay and a non-specific assay were performed. In the specific assay, the SciTox™ response of the sensor was tested and compared between the presence and absence of the specific analytes (Ag^{+}/Cu^{2+}). In the non-specific assay, the response of the sensors was tested and compared between the presence and absence of a non-specific analyte magnesium (Mg). In the presence of either $CuSO_4$ or $AgNO_3$, the Δ*nuoA* pSong11 sensor contributed to a significantly high SciTox™ signal compared to the untreated counterparts (Figure 5.7). This result showed that the presence of either Ag^{+} or Cu^{2+} cause increased cell respiratory activity by induction of the *nuoA* expression, and thereby significantly increase the SciTox™ signal. There was no significantly higher SciTox™ response detected in the presence of $MgSO_4$ (Figure 5.7). This control assay combined with the results from the specific assays confirmed that the Δ*nuoA* pSong11 bioassay responds specifically to the heavy metals Ag^{+} and Cu^{2+}.

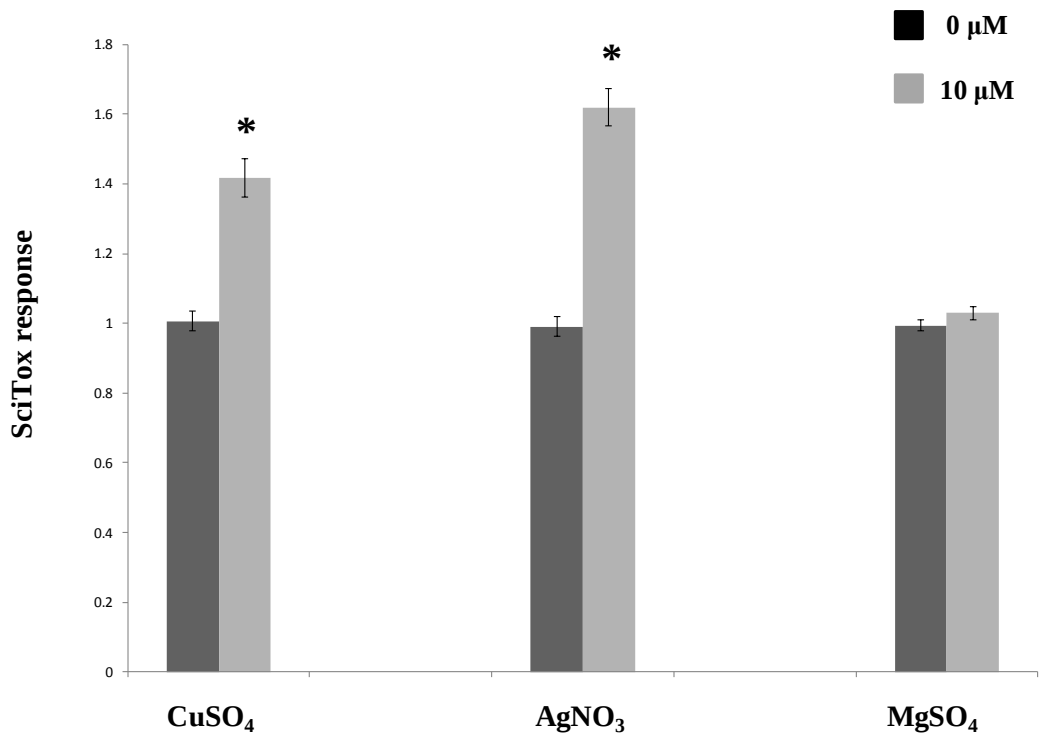

Figure 5.7. Comparison of SciTox^{TM} response of *ΔnuoA* pSong11 assay between the presence and absence of specific (Ag^{+} / Cu^{2+}) and non-specific (Mg^{2+}) analytes. The name of the analyte is given below the corresponding data bar. *: significantly different between 0 and 10 µM of the same analyte in a paired test at $P < 0.001$. n=5, error bars are standard error.

5.3.8.3 Unmodified *E. coli* SciTox^{TM} assay to Ag^{+}/Cu^{2+}

In order to confirm the sensitivity of Δ*nuoA* pSong11 bioassay, the unmodified *E. coli* K12 BW25113 (does not contain pSong11 plasmid) and Δ*nuoA* strain (does not contain pSong11 plasmid) were used as negative controls, and their SciTox^{TM} response were tested and compared between the presence and absence of either Ag^{+} or Cu^{2+} (Table. 5.2).

Table 5.2. Comparison of statistical differences of both *E. coli* BW25113 and Δ*nuoA* to both $CuSO_4$ and $AgNO_3$, between 0 and 10 μM.

Strains	Targets	Concentrations (μM)		P values	Significance	Replicates
		0	10			
		Mean of SciTox™ related signals				
***E. coli* BW25113**	**$AgNO_3$**	1.00	1.03	0.24	No	5
	$CuSO_4$	1.00	1.05	0.15	No	5
Δ*nuoA*	**$AgNO_3$**	1.00	0.95	0.10	No	5
	$CuSO_4$	1.00	1.04	0.13	No	5

Both unmodified *E. coli* strains did not produce a significant increase in the SciTox™ signal in the presence of either Ag^+ or Cu^{2+}. The result indicated that unmodified *E. coli* assay could not detect Ag^+ and/or Cu^{2+} at sub-inhibitory levels.

5.4 Discussion

In order to discover genes suitable for use in the SciTox™ assay, approximately 4000 single-gene knock-out mutants comprising the Keio *E. coli* collection (Baba et al. 2006) were tested (Weld et al. 2010). Three genes involved the *nuoA*, *ompF* and *selA* genes were selected as candidate reporter genes because they contribute to different cellular processes and loss of each of gene contributed to a significant reduction in of the SciTox™ signal compared to a non-mutated *E. coli* strain (Weld et al. 2010).

The *Tn10 tetA* promoter was chosen as our inducible promoter for antibiotic detection because it is a well characterized promoter that is strongly repressed and is specifically induced at low Tet concentrations (Flache et al. 1992; Gatz and Quail 1988). To test its functioning and response to Tet, the *tetA* promoter was transferred into pBluescript, fused to the *lacZ* reporter gene, replacing the *lac* promoter. The *tetA*

has been used as an inducible-promoter element for construction of genetically modified biosensors which were used to detect the antibiotic Tet in previous studies (Obana and Nishino 1990; Virolainen et al. 2008). The *lacZ* gene acts as a reporter gene which was widely used in blue-white colour assay. By using Top10f' pSong8, the presence of Tet could be detected by a visible blue colour. The assay confirmed that our *tetA* promoter construct responded to Tet. Additionally, to optimize Tet-induction, the growth phase of sensor cells and Tet-inducible periods were tested and determined as the early stationary-phase cells (Table. 5.1) and 90 mins induction period, respectively.

In order to ensure that the specificity and sensitivity of SciTox^{TM} assays could be improved based on gene induction in response to Tet, the unmodified *E. coli* acted as a negative control and its response to Tet was tested in SciTox^{TM} assay prior to the genetically modified *E. coli* bioassays. However, unmodified *E. coli* assay responded in a sensitive dose-dependent manner to Tet at sub-inhibitory levels when using stationary phase cells (Figure 5.2 A). The mode of action of Tet is that it binds to bacterial ribosomes and stops protein synthesis, thereby inhibiting cell growth (Speer et al. 1992). In unmodified *E. coli* assay, the stationary phase cells were not likely to be growing during the assay and therefore there is likely to be less inhibitory effect of Tet on the cells than at the exponential phase. The stimulatory effect of Tet on the SciTox^{TM} assay of stationary phase cells was, however, unexpected and cannot be explained.

The stationary phase cells are commonly used in unmodified SciTox^{TM} rapid toxicity assays (Pasco et al. 2004; Pasco et al. 2008; Pasco et al. 2001; Tizzard et al. 2004). Additionally, the comparisons between stationary phase and exponential phase cells in *lacZ*-based blue-white colour assay indicated that stationary phase cells gave a more sensitive response than exponential phase cells (Table 5.1). However, stationary phase cells seemed not suitable for use in the genetically modified SciTox^{TM} bioassays due to the unexplained dose-dependent response to Tet at sub-inhibitory levels in the negative control (Figure 5.2 A). In order to avoid the unexpected sensitive response to Tet, the unmodified *E. coli* assay was repeated by using exponential phase cells, which was maintained in LB culture medium. By using exponential phase cells, unmodified *E. coli* assay did not respond to Tet in a dose-dependent manner at Tet concentrations below sub-inhibitory levels, whereas loss of SciTox^{TM} signal at high

concentrations of Tet was due to the toxicity of Tet (Figure 5.2 B). In order to create a bio-assay that can identify and quantify Tet at concentrations below inhibitory levels, the SciTox^{TM} assay was re-engineered based on four separate strategies. They were all based on gene induction in response to Tet.

The *lacZ* gene is a well known reporter gene used in blue-white colour assay. In a recent study, *lacZ* has been used in an amperometric whole-cell based biosensor in yeast to detect and quantify the metal Cu (Lehmann et al. 2000; Tag et al. 2007). The product of *lacZ*, β-galactosidase is able to metabolize lactose to galactose and glucose. Based on this, the *lacZ* gene was selected as a candidate for use as a reporter gene in a SciTox^{TM} biosensor for amperometric detection and quantification of Tet. However, *lacZ*-based bioassay did not respond to Tet in a dose-dependent manner. The reason is not known. It might be due to the low expression of *lacZ* during the exponential growth-phase.

In order to link the overall redox activity of respiratory enzymes to the presence of Tet, *selA* was fused to *tetA* promoter and transformed into the Δ*selA* strain. However, the *selA*-based biosensors did not exhibit an improved response to Tet, compared with the unmodified *E. coli* assay (Figure 5.2 B). It might be due to the low turnover rate and expression of selenoproteins during the SciTox^{TM} assay. Thus, low levels of selenoprotein may have no detectable impact on the respiration rate.

The ompF protein encoded by the *ompF* gene, forms a large porin channel involved in passive diffusion across the outer membrane in Gram-negative bacteria, including *E. coli* (Bekhit et al. 2011; Garcia-Gimenez et al. 2011; Housden et al. 2010). By using *ompF* as the reporter gene, the entry efficiency of mediator (KFCIII) across sensors outer membrane due to passive diffusion, was linked to the presence of Tet. The *ΔompF* pSimon assay responded to Tet in a sensitive dose-dependent manner (Figure 5.3). The DL of Tet by the *ΔompF* pSimon assay was 0.013 μg ml^{-1}, which was almost one order of magnitude more sensitive than the MRL of EU (Virolainen et al. 2008). Additionally, the *ΔompF* pSimon assay was at least as sensitive as some of the visual reporter genes (*lacZ*, *gfp* and *lux*) based whole-cell biosensors (Hansen and Sorensen 2000; Virolainen et al. 2008) and very close to a more recent luminescent-based *E. coli* whole cell biosensor (Pikkemaat et al. 2010).

The *nuo* operon encodes the enzyme NADH dehydrogenase I, which catalyses the transfer of electrons from NADH into the start of the respiratory chain (Wackwitz et al. 2000), and functions in both anaerobic and aerobic conditions (Calhoun et al. 1993). The *nuoA*-based bioassay showed a more sensitive dose-dependent manner to Tet (Figure 5.5) than *ompF*-based assay (Figure 5.3). The DL of Tet by the *nuoA*-based bioassay was 0.0026 μg ml^{-1} (Figure 5.5) which was considerably much lower than the MRL of EU (Virolainen et al. 2008). The *nuoA*-based SciTox^{TM} bioassay also showed a higher sensitivity to Tet (about one order of magnitude), compared to most visual reporter gene-based genetically engineered whole-cell bioassays, including *gfp* (Hansen and Sorensen 2000; Scaria et al. 2009; Shen et al. 2011), *luxCDABE* (Hansen and Sorensen 2000; Pikkemaat et al. 2010; Virolainen et al. 2008) and *lacZ* (Hansen and Sorensen 2000).

In contrast to other bioassays, the *nuoA*-based assay also gave a lower DL for Tet. For example, an *E. coli* electrochemical biosensor has been reported to detect Tet at 0.025 μg ml^{-1} based on the measurement of carbon dioxide production rate in relation to inhibition of microbial growth (Pellegrini et al. 2004). The DL of Tet determined by a recent surface Plasmon resonance (SPR) biosensor was at 0.015 μg ml^{-1} (Moeller et al. 2007). In a more recent study, an improved *Bacillus cereus* inhibition bioassay could detect Tet as low as 0.109 μg ml^{-1} (Nagel et al. 2011). Moreover, the DL of Tet determined by *nuoA*-based SciTox^{TM} assay was lower than the DL of liquid chromatographic-ultraviolet (LC-UV) methods (Andersen et al. 2005; Kaale et al. 2008; Lee et al. 2007; Zhou et al. 2009) and very close to the DL of LC-mass spectrometry (LC-MS/MS) (Granelli et al. 2009; Xu et al. 2008; Yue et al. 2006) and high performance liquid chromatography (HPLC) (Jing et al. 2011; Shalaby et al. 2011).

In order to confirm that *nuoA*-based SciTox^{TM} assay could be used for detecting a wide range of environmental compounds, the *nuoA* gene was then fused to an Ag/Cu-inducible *copA* promoter. The Δ*nuoA* pSong11 assay responded in a "mountain" shaped dose-dependent manner to Ag (Figure 5.6 A). However, the Δ*nuoA* pSong11 assay responded to Cu^{2+} in a distinct dose-dependent manner (Figure 5.6 B) compared with the response to Ag^{+}. A persistent increased SciTox^{TM} signal was achieved within the Cu^{2+} concentrations of 10^{-2} to 10^{2} μM. The different dose-dependent profiles contributed by Ag^{+} and Cu^{2+} might be due to the different mechanisms of activation

of the *copA* promoter by the two different metal ions. The *copA* promoter is hypersensitive to Ag^{+} and Cu^{+} and the response to Cu^{2+} *in vivo* is a measure of conversion of Cu^{2+} to Cu^{+} (Stoyanov et al. 2001).

My results also agreed with a previous study (Stoyanov et al. 2001). The SciTox^{TM} response was higher than 1.6 at Ag^{+} of 10 μM whereas the response was below 1.5 at Cu^{2+} of 100 μM. These results indicated that *copA* promoter is highly responsive to Ag^{+} than Cu^{2+}. Therefore, the response of Δ*nuoA* pSong11 to Ag^{+} declined at $AgNO_3$ concentration above 10 μM (Figure 5.6 A) was caused by loss function of proteins, due to the formation of inclusion bodies. The SciTox^{TM} response to Cu^{2+} could increase from 10^{-2} to 10^{2} μM because the *copA* promoter had not reached the optimal activation yet.

There are a number of enzyme-based biosensors used to analyse heavy metals (e.g. Ag/Cu) based on activation or inhibition of their activities (Corbisier et al. 1999; Fennouh et al. 1998; Verma and Singh 2005). For example an inhibition of immobilized urease study combined with an ion-selective field effect transistor (ISFET) transducer determined the IC_{50} (the metal ion concentration that inhibits by 50%) of Ag^{+} and Cu^{2+} at 0.2 and 5.0 μM respectively (Volotovsky et al. 1997). To my knowledge, only one group has developed an amperometric yeast biosensor to detect the heavy metal Cu^{2+} (Lehmann et al. 2000). The sensor was constructed by fusing a Cu-inducible *CUP1* promoter to *lacZ* reporter gene. The sensor could detect Cu^{2+} at a sensing range of 0.5 – 2.0 mM. The assay was further studied by using flow injection analysis (FIA), and the sensing detection range of Cu^{2+} was 1.5 -7 mg L^{-1} (23.4 – 109.4 μM) (Tag et al. 2007). In contrast, the Δ*nuoA* pSong11 bioassay could detect Cu^{2+} at a wider sensing range of 0.1 – 100 μM (Figure 5.8 B).

In this study, two genetically modified *E. coli* bioassays were developed and responded to Tet in a specific dose-dependent manner at sub-inhibitory level. Of the two assays, the specificity and sensitivity to Tet was largely improved by using *nuoA* as a reporter gene. Furthermore, *nuoA* could be used as a reporter gene to specifically and with higher sensitivity to detect other environmental compounds except Tet, by fusing with different inducible promoters. This is the first time that a key respiratory gene was used in a whole-cell based biosensor to amperometrically detect and quantify Tet and metal ions. However, these bioassays still had two limitations.

Firstly, a loss of SciTox^{TM} signal was recorded at high Tet concentrations which might be due to over-expression of the transgene leading to formation of protein inclusion bodies. This problem reduced the effectiveness of the bioassays. Secondly, high background readings were observed at zero analyte, in both of SciTox^{TM} amperometry assay and blue-white colour assay. This limitation might have been caused by basal expression of the transgene, thereby lowering Tet detection sensitivity of the bioassay. In the next chapter, an attempt was made to solve these problems.

5.5 Conclusions

Four separate genetically modified *E. coli* biosensors were developed and an attempt was made to specifically and with a high sensitivity to identify and quantify Tet at concentrations below inhibitory levels. They were all based on gene induction in response to Tet. Two assays (*ompF*- and *nuoA*-based assays) responded specifically to Tet in a dose-dependent manner, more sensitive than the unmodified SciTox^{TM} assay and other similar DNA assays (Ertl et al. 2000). These two assays may provide a strategy that could allow the SciTox^{TM} assay to be developed as a sensitive and specific bioassay. However, the sensitivity of SciTox^{TM} assay to specific analyte was not improved by using *lacZ* and *selA* as reporter genes. The *nuoA*-based assay showed a higher sensitivity than *ompF*-based assay. This assay is at least as sensitive as other whole cell biosensors reported to detect Tet using bioluminescence or fluorescence reporter genes (Pellegrini et al. 2004; Scaria et al. 2009; Virolainen et al. 2008). Additionally, *nuoA* could also be used as a reporter gene to specifically and with a high sensitivity to detect other environmental compounds (e.g. Ag/Cu) in SciTox^{TM} system. To our knowledge, this is the first time this gene, or any other respiratory reporter gene, has been used as the reporter gene for an amperometric biosensor. However, two limitations, the loss of SciTox^{TM} signal at high concentrations of analyte and high basal reading at zero analyte, need to be improved in a future study.

CHAPTER 6: THE IMPROVEMENT OF *NUOA* ASSAY FOR TET MEASUREMENT

6.1 Introduction

In our previous study (Chapter 5), four different genetically modified *E. coli* bioassays were developed to detect and quantify the antibiotic Tet in the SciTox^{TM} assay. All four sensors were constructed based on gene induction in response to Tet. Both the *ompF*- and *nuoA*-based bioassays could identify and quantify Tet at sub-inhibitory levels. Of the two assays, the *nuoA*-based bioassay (containing plasmid pSong10) was more sensitive to Tet. In contrast to other studies, the *nuoA*-based bioassay is at least as sensitive as visual reporter gene-based (*lacZ*, *gfp* and *lux*) whole cell biosensors (Hansen and Sorensen 2000, 2001; Pikkemaat et al. 2010; Scaria et al. 2009; Shen et al. 2011; Virolainen et al. 2008). Additionally, *nuoA* could also be used as a reporter gene to specifically and sensitively detect other environmental compounds in SciTox^{TM} assay (e.g. Cu and Ag) rather than Tet, simply by changing the specific inducible promoter (Chapter 5).

Two aspects of the assay needed to be improved. Firstly, the SciTox^{TM} current increases as Tet concentrations increase but beyond a certain concentration the SciTox^{TM} current declines rapidly, dropping below the current detected at zero Tet concentration. This limitation reduces the effectiveness of the assay. Secondly, the *nuoA*-based assay has a relatively high background current. In the absence of Tet, the *nuoA* assay showed a significantly higher SciTox^{TM} signal than the *nuoA* knockout *E. coli* strain without the biosensor transgene construct. This limitation might play a role in lowering the Tet detection sensitivity (DL). Both these limitations were also observed in the *ompF*-based bioassay. Therefore, this study was directed at overcoming these two limitations, thereby improving the SciTox^{TM} response (DL and detection range) of *nuoA* bioassay to Tet. The *nuoA*-based bioassay was used as a model system to find methods to overcome these problems because of its high sensitivity compared to the *ompF*-based bioassay.

Proteins over-expressed in *E. coli* often accumulate in the form of inclusion bodies, which are insoluble, inactive aggregates of misfolded proteins (Buchanan 1999; Kiefer 2003). There are many factors that may influence the formation of protein

inclusion bodies, including culture media, growth temperature, production rate (e.g. gene dosage, promoter strength, mRNA stability and codon usage), and the availability of heat-shock chaperones (Chen et al. 2003; Ventura and Villaverde 2006). Newly synthesized proteins readily form inclusion bodies at high growth temperature (37 ˚C) due to a high protein synthesis rate (Chen et al. 2003). By lowering the growth temperature to 15 ˚C, the formation of protein inclusion body can be significantly reduced (Lethanh et al. 2005). Similarly, it should be possible to reduce over-expression by reducing the transgene copy number. In our previous study (Chapter 5), transgene constructs were carried on the high-copy number, pBluescript-derived, plasmids. In this work the plasmid pBR322 was used as the vector to carry the transgene (fusion of *tetA* promoter with *nuoA* gene [*PtetA::nuoA*]), instead of pBluescript. The pBR322 is a low-copy number plasmid that has a copy number of 15 – 20 copies / chromosome (Mayer 1995).

The high background SciTox^{TM} current detected at zero Tet concentration from the biosensor strain might be caused by reporter gene basal expression due to incomplete turn off of the *tetA* promoter. The high background reporter gene expression was also likely to be exacerbated by the many transgene copies present in each cell on the high-copy number plasmid. Therefore we also looked at the possibility of reducing background SciTox^{TM} signal by placing the transgene on a low-copy number plasmid.

6.2 Method

Construction of pLBRSong10: the low-copy number plasmid pBR322 was digested by *SalI* and *BamHI*, and ligated to the transgene *PtetA*::*nuoA* amplified from plasmid pSong10 by PCR using primers 5’ – ATCA **GTCGAC** AAT GGG AAT TGA CGT TCC TTC – 3’ and 5’ – ATAG **GGATCC** TCA ACC GGG ATG AAT TTA TCG – 3’ also digested with *SalI* and *BamHI* restriction enzymes.

Conjugation (transfer Tet-resistant f’ plasmid from Top10f’ to *nuoA* knock-out *E. coli* strains): Both *E. coli* Top10f’ (Tet-resistant) and *nuoA* knockout *E. coli* (Kan-resistant) strains were grown in separate flasks with 50 ml of LB broth and

appropriate antibiotics, at 37 °C, 200 rpm overnight. 10 ml of each overnight culture was transferred into a new flask. The mixture of the two overnight cultures was then incubated together at 37 °C, 200 rpm for 4-5 hours. 100 µl of the mixture was spread onto a LB agar plate which was coated by both Tet/Kan. The plate was then incubated at 37 °C overnight (Raya and Jabri 2008).

The SciTox™ assay (exponential-phase cell): exactly same as described in **Section (5.2)**.

The low temperature SciTox™ assay: 1 ml of exponential-phase cells was incubated with 1 µl of variable concentrations of Tet. The plates were incubated at 15 °C, 200 rpm for 6 hrs; then 300 µl of 250 mM KFCIII was added to each well.

6.3 Result

6.3.1. Tet toxicity assay

Part of the reduction in SciTox™ signal at high analyte concentrations could be due to a toxic effect since some of the transgene biosensor strains developed were not specifically resistant to the analyte. Therefore we transferred Tet-resistance (f' plasmid) into the *nuoA* knockout *E. coli* strain (*ΔnuoA* f'). The *ΔnuoA* f' strain was then exposed to a broad concentration range of Tet in SciTox™ assay to confirm its Tet-resistance. Without the f' plasmid, the strains were affected by toxicity at Tet above 0.1 µg ml^{-1} (Figure 5.2 B). In contrast, there was no observed toxic effect detected in neither *E. coli* Top10f' nor *ΔnuoA* f' assays at Tet below 10 µg ml^{-1} in (Figure 6.1).

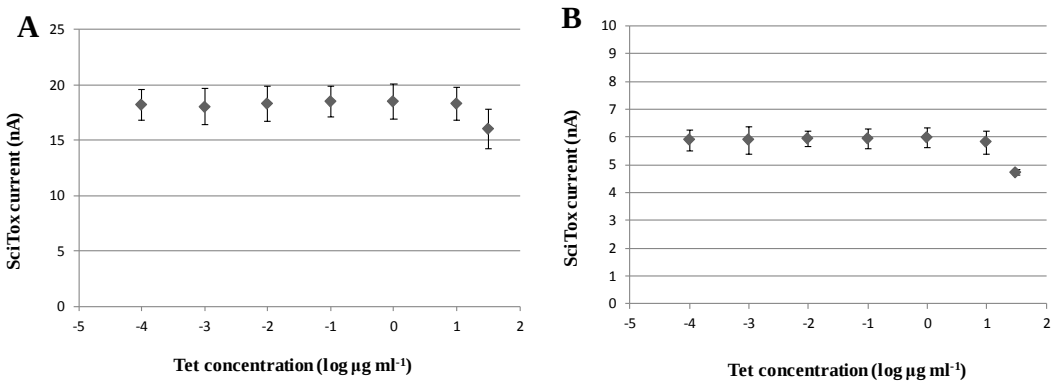

Figure 6.1. SciTox^{TM} response (Mean + SD) of two Tet-resistant *E. coli* strains, exposed to different Tet concentrations in water sample. A: *E. coli* Top10f' assay; B: *ΔnuoA* f' assay. Tet concentration is in log scale.

6.3.2. Tet-resistant *nuoA* assay *(ΔnuoA* f'pSong10 assay)

The response of *ΔnuoA* f'pSong10 sensor to a broad range of concentrations of Tet was tested in the SciTox^{TM} assay. The assay responded to Tet in a dose-dependent manner from 0.001 to 0.1 µg ml^{-1} Tet and the SciTox^{TM} signal declined at Tet above 0.1 µg ml^{-1} (Figure 6.2). As this strain carried the f' plasmid encoding Tet resistance, the loss of SciTox^{TM} signal (at Tet from 0.1 – 10 µg ml^{-1}) was probably not due to toxicity of Tet (Figure 6.1) and therefore we suspected that over-expression may have caused it.

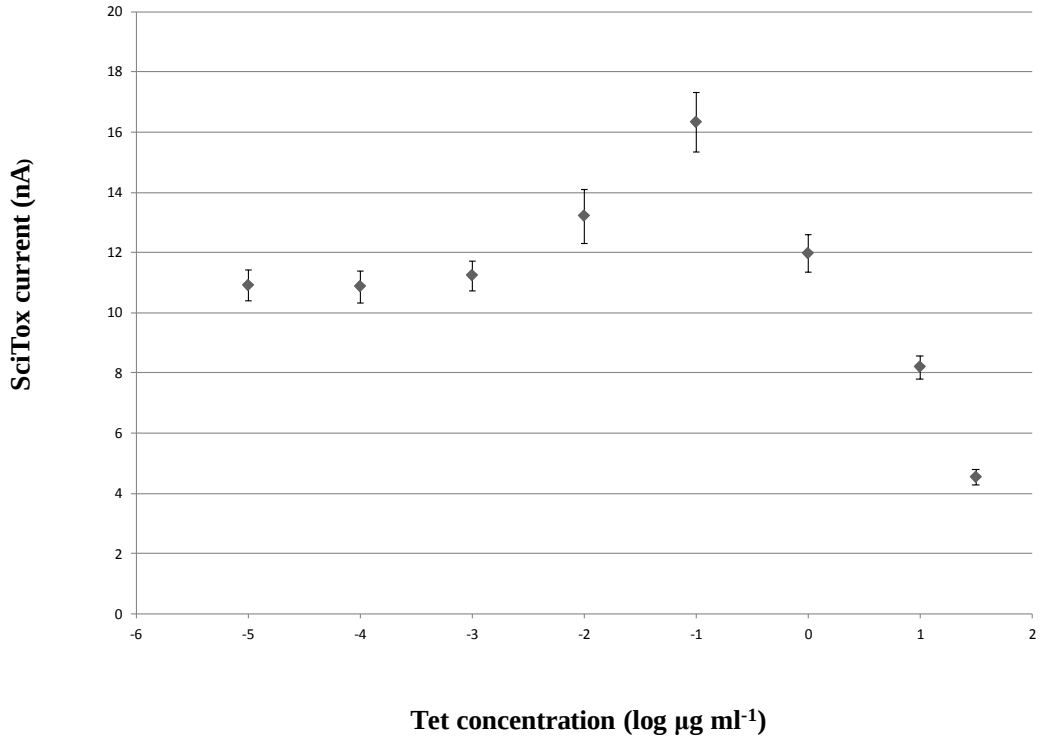

Figure 6.2. SciTox^{TM} response (Mean + SD) of *ΔnuoA* f'pSong10 strain, exposed to different Tet concentrations in water sample. Tet concentration is in log scale.

6.3.3. Low temperature *nuoA* assay (*ΔnuoA* f'pSong10)

In order to test whether the optimal induction concentration of Tet could be improved by overcoming both Tet toxicity and gene over-expression, a low temperature assay was tested using Tet-resistant *nuoA* biosensor (*ΔnuoA* f'pSong10). For this assay, the maximum induction Tet concentration was increased to 1 µg ml^{-1} (Figure 6.3). The Tet detection range was from 0.001 to 1 µg ml^{-1}. However, the SciTox^{TM} response still declined at non-toxic Tet levels (Figure 6.3).

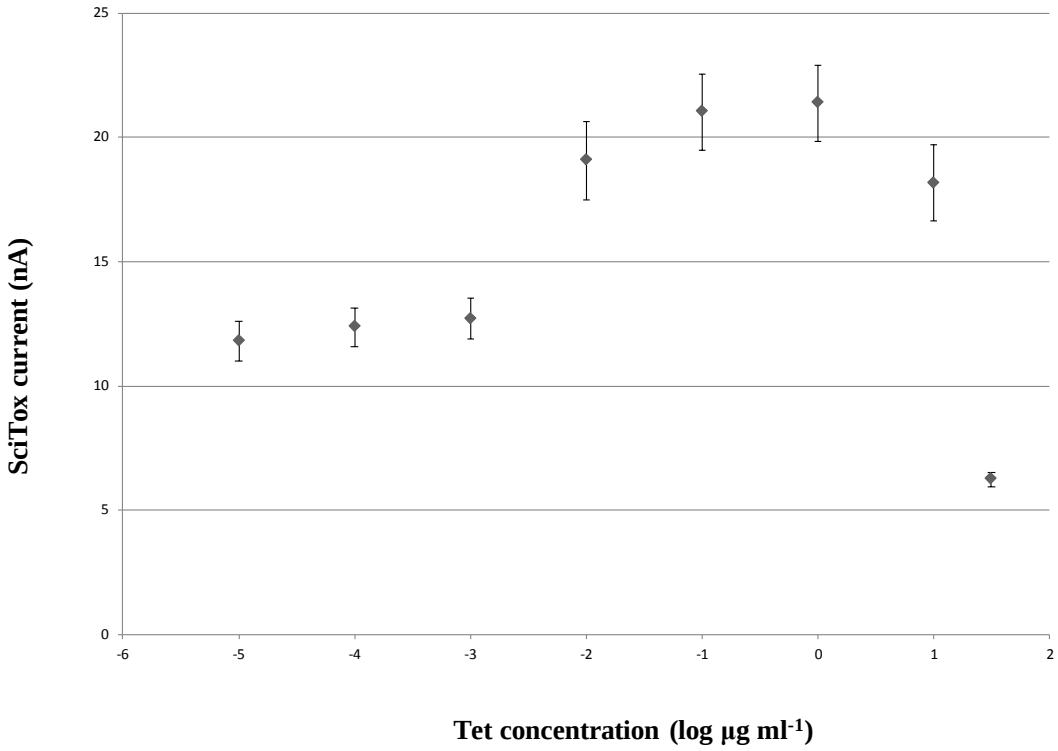

Figure 6.3. SciTox^{TM} response (Mean + SD) of *ΔnuoA* f'pSong10 strain, exposed to different Tet concentrations at 15 ℃ in water sample. Tet concentration is in log scale.

6.3.4. Low copy-number *nuoA* assay (*ΔnuoA* f'pLBRSong10 assay)

If the decline in the SciTox^{TM} response could be reduced by lowering the copy number of the reporter gene was then tested. A low-copy number plasmid, pBR322 was used to carry the transgene (*PtetA::nuoA*) to reduce gene over-expression in a Tet-resistant *nuoA* strain carrying the f' plasmid. The response of the sensor to a broad range of Tet concentrations was then tested in the SciTox^{TM} assay. The sensitivity of this assay to Tet was more (Figure 6.4) than in other *nuoA* bioassays (Figure 6.2 and 6.3). The DL of Tet determined by this assay was 0.00023 µg ml^{-1} (Figure 6.4 B). This result might indicate that the sensitivity of *nuoA* bioassay could be improved by using low copy-number plasmid. For this assay, the optimal Tet induction concentration was 1 ug ml^{-1}, and the detection range was from 0.0001 – 1 ug ml^{-1} (Figure 6.4 A). However, similar to low temperature *ΔnuoA* f'pSong10 assay (Figure

6.3), the SciTox^{TM} signal declined at Tet above 1 µg ml^{-1} (Figure 6.4). A combination of a low temperature assay with the Tet-resistant low-copy number strain was tested but that did not bring about a better response. When the low-copy number assays were performed with strains that were not Tet resistant, the signal declined after 0.1 µg ml^{-1} Tet indicating that toxicity could also affect the reporter gene function (Figure 6.5).

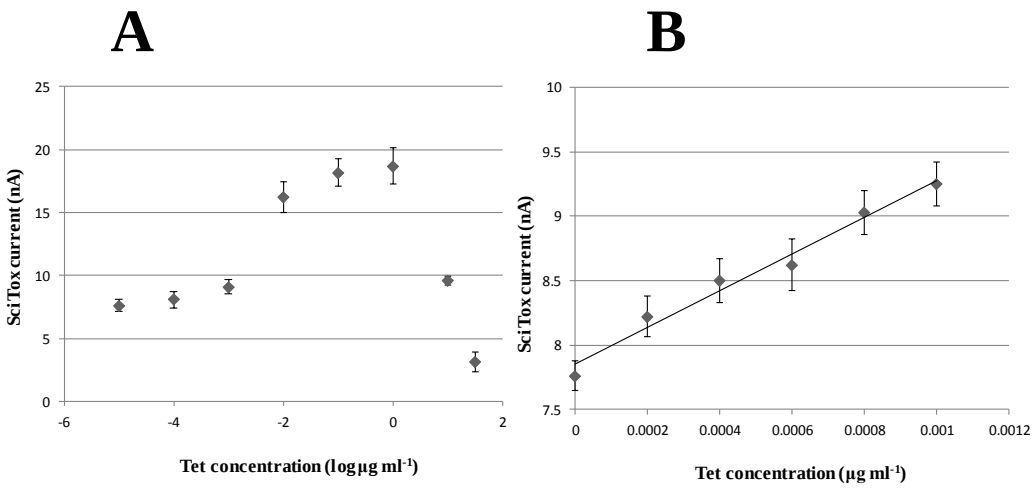

Figure 6.4. SciTox^{TM} response (Mean + SD) of *ΔnuoA* f'pLBRSong10 strain, exposed to different Tet concentrations in water sample. A: broad concentration range. B: dose-dependent range. The fitted linear regression line is y = 1424.30x + 7.90, R^2 = 0.98 for the dose-dependent data range. Tet concentration in A is in log scale and in B is in linear scale.

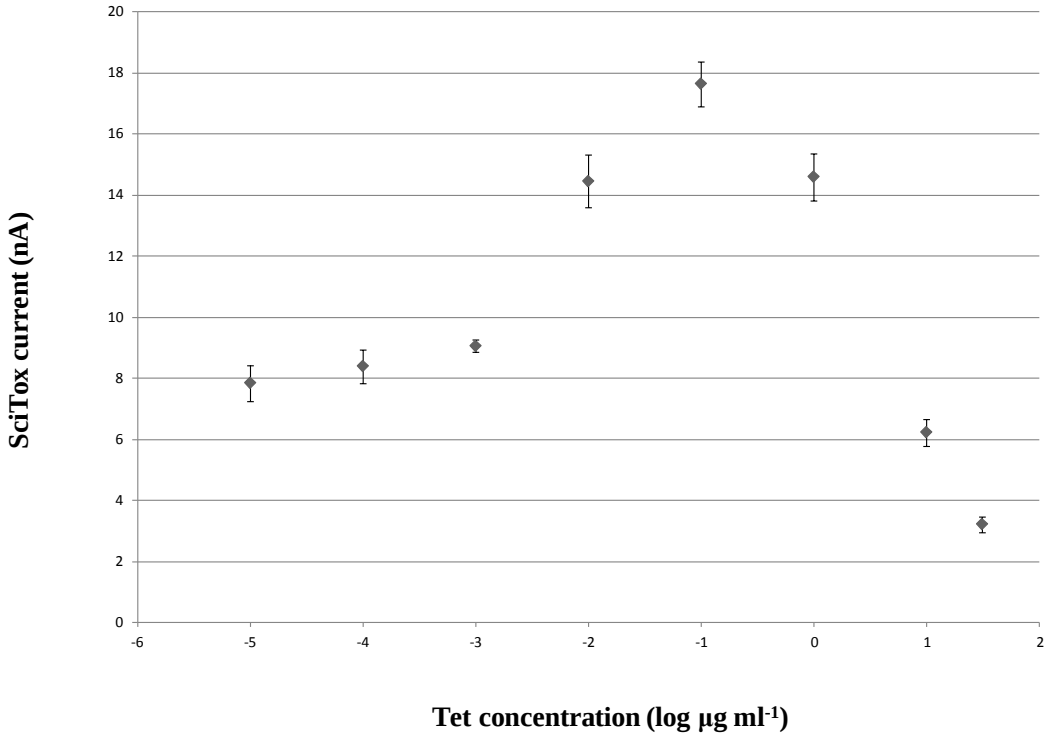

Figure 6.5. SciTox^{TM} response (Mean + SD) of *ΔnuoA* pLBRSong10 strain, exposed to different Tet concentrations in water sample. Tet concentration is in log scale.

6.3.5. Tet and mediator (KFCIII) interaction assay

In order to confirm that the decline in SciTox^{TM} signal at high concentration of Tet was not due to the interaction between Tet and the mediator (KFCIII), the response of KFCIII to a broad range of concentrations of Tet was tested in the SciTox^{TM} assay. KFCIII did not respond to Tet in a dose-response manner. This assay confirmed that there was no interaction between the mediator (KFCIII) and the analyte (Tet).

6.3.6. Comparison of gene basal expression between high-copy number and low-copy number *nuoA* assays

In order to confirm that both of gene basal expression and over-expression could be reduced by using low-copy number plasmid, the SciTox^{TM} currents of four different *E.*

coli assays were measured and compared at two different concentrations of Tet. Both unmodified *E. coli* and *nuoA* knockout *E. coli* assays were used as controls. At zero Tet, there was still a significant ($P<0.05$) level of background current observed in low-copy number *nuoA* assay (Figure 6.6). Despite this, the SciTox^{TM} background reading was significantly ($P<0.005$) reduced, in contrast to the high-copy number *nuoA* assay (Figure 6.6). At a Tet concentration of 0.1 μg ml^{-1}, low-copy number *nuoA* assay showed significantly ($P<0.05$) greater induced SciTox^{TM} current than high-copy number *nuoA* assay (Figure 6.6). This result indicates that there are more catalytically active proteins produced by *nuoA* gene expression in the low-copy number assay due to the reduction of reporter gene over-expression. The assay confirmed that both basal expression and over-expression of the gene could be reduced by using a low-copy number plasmid.

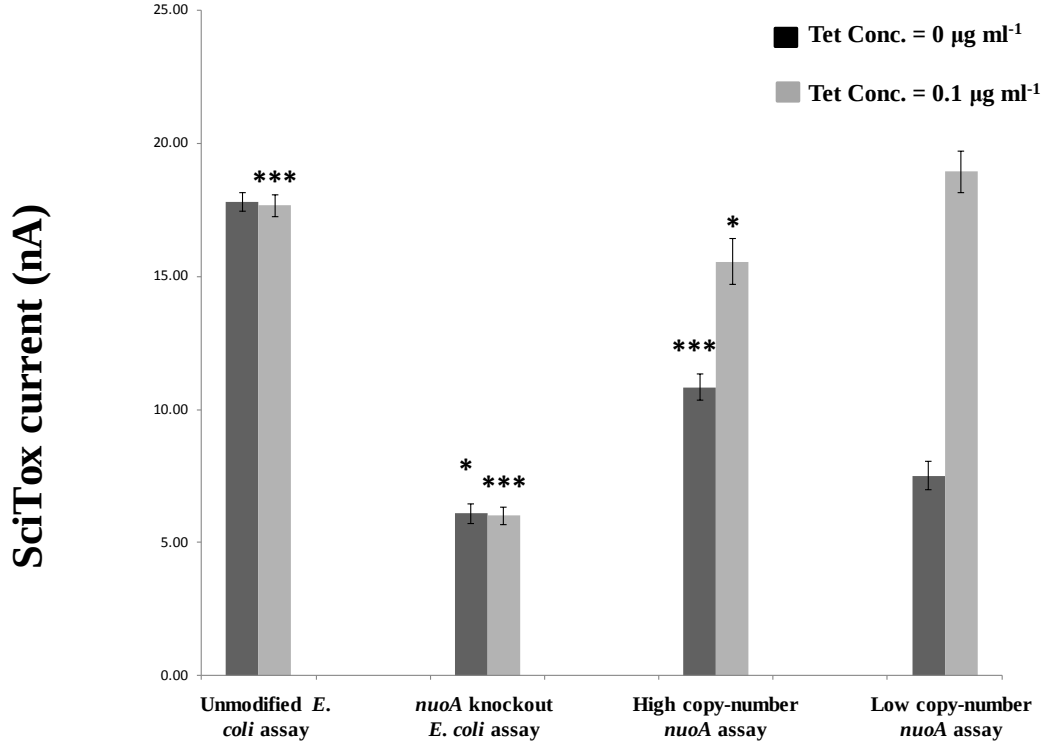

Figure 6.6. Amperometric currents comparison between four different *E. coli* assays, when exposed to two different concentrations of Tet in SciTox^{TM} assay. The name of different *E. coli* strains is given below the corresponding data bar. *: significantly different from low copy-number *nuoA* assay. The number of stars (*) indicates the

significance level. *: P<0.05; **: P<0.01; ***: P<0.005. The significant difference was determined in paired test. n=6, error bars are standard error.

6.4 Discussion

The decrease in SciTox^{TM} signal at high analyte concentrations makes the transgenic SciTox^{TM} assay ambiguous since at high analyte concentrations the assay can return a signal that is lower than the signal produced at lower analyte concentrations. In practical applications, the ambiguity could be removed by testing samples at more than one dilution, though ideally that would not be necessary. The mechanism of signal inhibition at higher concentrations might also reduce the magnitude of the response at lower concentrations and that may limit the assay's maximal response, reducing the effectiveness of the assay. Consequently, we tested strategies to overcome this limitation.

The test analyte, Tet, is an antibiotic that is toxic to bacterial cells. The *Tn10* Tet resistance gene carried on the f plasmid conferred resistance to Tet in our strains to more than 10 μg ml^{-1} (Figure 6.1). On its own, however, Tet resistance did not significantly improve the assay (Figure 6.2). Another factor that might decrease the signal at high analyte concentrations is transgene over-expression, leading to the formation of inclusion bodies which is a common limitation of recombinant protein production in bacteria (Ventura and Villaverde 2006), especially in *E. coli* (Mayer and Buchner 2004). We tried two strategies to reduce over-expression: gene induction at low temperature; and placing the inducible transgene on a low copy number plasmid. On their own, these strategies did not produce appreciable improvements in the assay (Figure 6.5). By combining strategies to reduce over-expression with Tet resistance, however, it was possible to increase the maximum SciTox^{TM} response and to significantly increase the SciTox^{TM} signal at higher analyte concentrations (Figure 6.3 and 6.4). The loss of signal at high analyte concentrations was likely at least in part due to a combination of toxicity of the analyte and loss of active *nuoA* protein due to over-expression of *nuoA* at high concentrations of the analyte.

Clearly it is advantageous, if the assay is to be used to measure toxic analytes at high concentrations, to provide the biosensor bacteria with a mechanism for resistance to

the analyte. The Tet resistance gene *tetA* on the f' plasmid encodes an efflux pump that pumps Tet out of the cell (Speer et al. 1992; Teo et al. 2002). It is surprising that expression of an efflux pump was not detrimental to the assay, as it should lead to a lower Tet concentration inside the cell and consequently lower *nuoA* gene induction. Presumably, there was not enough time available during the induction period for the efflux pump to be expressed and to reduce the intracellular Tet concentration sufficiently to noticeably reduce *nuoA* induction. This suggests that efflux pumps, which are quite a common mode of toxin resistance in bacteria, are compatible with gene-induction, whole-cell biosensors.

There are a number of different methods that could be used alternatively to reduce the formation of protein inclusion bodies (Chen et al. 2003; Ventura and Villaverde 2006) thereby improving the response of our bioassays to target analytes. For example, decreasing transgene promoter transcription efficiency might reduce gene over-expression. The conserved hexamers located at positions -35 (TTGACA) and -10 (TATAAT) in the promoter play an essential role in the initiation of transcription by RNA polymerase (Babb et al. 2004). It has been reported that mutations which increased identity to the consensus hexamers, generally led to an increased transcription efficiency of the promoter (McCracken and Timms 1999). Therefore by reducing the sequence identity to the consensus hexamers, the strength of the promoter might be reduced. Moreover, the Shine-Dalgarno sequence (SDS) (AGGAGGT) that is normally located at 6-8 bps upstream of the start codon, is complementary to the sequence in the 3'-terminal of the 16S ribosomal RNA (16S rRNA) (Shine and Dalgarno 1974). SDS can help recruit the ribosome to the mRNA to initiate protein synthesis (Starmer et al. 2006). Mutations in SDS might reduce the efficiency of protein expression thereby reducing formation of inclusion bodies.

To reduce the yield of transgene product further, the transgene could be integrated into the chromosome, thereby reducing the copy number down to 1 copy/chromosome (Alonso et al. 2003). Additionally, a relatively low recombinant protein expression system (e.g. yeast) could be used instead of *E. coli* (Cantrell 2003) to reduce the yields of protein products. Over-expression of chaperons (e.g. DnaK/DnaJ) along with the reporter gene could increase the folding efficiency of target protein (Chen et al. 2003), thereby preventing protein aggregation. Also, expression of heat shock proteins (e.g. Hsp70/IbpA/IbpB) might reduce inactivation and aggregation of the target

proteins and may also facilitate their subsequently refolding by chaperons (Jurgen et al. 2010).

The background SciTox^{TM} signal from uninduced transgenic cells was significantly higher than the background SciTox^{TM} signal obtained from the parent *nuoA* strain without the inducible transgene construct (Figure 6). This suggests that the inducible transgene was not fully repressed even when there was no target analyte present. The high background transgene expression was probably exacerbated by the fact that the construct was carried on a high copy number plasmid. Once the transgene was transferred to a low copy number plasmid, the background expression was significantly reduced.

Despite this, even with a low-copy number plasmid, the background SciTox^{TM} signal from uninduced transgenic cells was marginally, but significantly, higher than the background SciTox^{TM} signal obtained from the parent *nuoA* strain without the inducible transgene construct (Figure 6). To further reduce the background expression of *nuoA*-based biosensor down to a similar level relative to its parent strain, four methods could be used due to improving TetR regulation. Firstly, increase of *tetR* promoters (P_Rs) strength could be useful. There are two promoters P_R1 and P_R2 involved in P_Rs. The *tetR* gene can be transcribed by either P_Rs (Meier et al. 1988). The expression of TetR can be improved by increase of P_Rs transcription efficiency. Moreover, the *Tn10* transposon contains two operators *O1* and *O2* (Oehmichen et al. 1984). Occupation of *O2* by TetR protein only could slightly repress P_R1, while occupation of *O1* by TetR could completely repress both P_R1 and P_R2 (Oehmichen et al. 1984). In the second method, mutations in the sequence of *O1* could reduce binding affinity between TetR and *O1*, thereby up-regulating the transcription of TetR from P_Rs. This method could be used to overcome gene over-expression as well. Thirdly, decrease of *tetA* promoter transcription efficiency could reduce the expression of reporter gene. Finally, the transgene could be integrated into the chromosome to further reduce the copy number.

6.5 Conclusions

The loss of SciTox^{TM} signal at high concentrations of the analyte (Tet) was at least partly caused by both analyte toxicity and reporter gene over-expression. By using low-copy number plasmid along with Tet-resistance, the detection limit and optimal induction of the assay was improved. The strategy of using an efflux pump that conferred resistance to the analyte by pumping it out of the cells was compatible with whole cell biosensors using an inducible reporter gene. Even with analyte resistance and low-copy number of the transgene, it was still not possible to stop the assay signal from declining below the background level at high analyte concentrations.

CHAPTER 7: GENERAL DISCUSSION AND FURTURE RESEARCH

7.1 General discussion

Pollution of soil and water caused by agriculture, industry (including war industry), combustion processes and accidental contamination with fuels, oils, solvents, and heavy metals is becoming an issue of immense global concern. The detection and quantification of environmental compounds is one of the important components in environmental monitoring. Some conventional methods, such as HPLC, have high precision and sensitivity, but are expensive and time-consuming. Therefore, inexpensive, rapid and sensitive methods which can detect and quantify environmental toxicants are being developed. These new methods include biosensors.

SciTox^{TM} rapid mediated DTA assay is a commercially available whole-cell microbial assay that measures toxicity through inhibition of bacterial respiration and is currently in commercial use to detect and quantify wastewater toxicity (Tizzard et al. 2004). This bacterial toxicity assay compares the difference in the current produced by microbial cells exposed to environmental samples with the current produced by unexposed cells (Pasco et al. 2005). However, SciTox^{TM} assay is confronted by two major constrains; the stability of whole cells and a lack of specificity to a target analyte. My research was based on overcoming these two limitations.

In the unmodified SciTox^{TM} toxicity assay, freshly cultured bacterial cells are used as biosensors. However, fresh cells require a three-step culture, which is time consuming. Recently, many studies have focused on using freeze-dried cells which offer both reliable and stable biosensing tools to monitor and detect toxicity (Choi and Gu 2003). However, none of the studies tested the shelf-life of freeze-dried cells and whether these could be used in toxicity tests. Therefore, in the first part of this research, I used freeze-dried cells in toxicity assay instead of fresh cells. Three different bacteria, *A. calcoaceticus*, *E. coli* and *P. putida* were chosen to investigate into the freeze-drying process and subsequently their use in the SciTox^{TM} toxicity assay. The efficiency of two different cryoprotectants (PEG and sucrose/Tween 80) and two storage temperatures (4 ˚C and -20 ˚C) were tested by measuring the cell viability and

determined the shelf-life of freeze-dried cells in SciTox^{TM} DTA assay at three different storage intervals; 1, 2 and 3 months. The dose-response and EC_{50} of pre-treated freeze-dried cells exposed to two different cryoprotectants and two storage temperatures at three storage periods exposed to 2,4/3,5-DCP, were determined by SciTox^{TM} toxicity assay, and compared with their freshly cultured counterparts. To better understand the effect of freeze-drying process on the viability of freeze-dried cells and the efficiency of cryoprotectants during the freeze-drying, the response of freeze-dried cells at zero-time to standard toxicants acted as the controls.

Irrespective of the cryoprotectant and storage temperature, all three freeze-dried bacterial strains after 1- and 2-month storage showed a similar dose-response profile to 2,4/3,5-DCP (Figure 4.2) in SciTox^{TM} toxicity assay, when compared with their fresh counterparts (Figure 4.1). However, the freeze-dried bacterial strains seemed to be more sensitive to the standard toxicants in SciTox^{TM} DTA assay following storage, as shown by lower the EC_{50} values to both standard toxicants as storage period increased (Table 4.2 and 4.3), relative to the EC_{50} values of zero-time freeze-dried counterparts (Table 4.1). After 3-month storage at 4 ˚C, both PEG pre-treated freeze-dried *A. calcoaceticus* and *E. coli* were not responsive and therefore could not be used in the SciTox^{TM} assay, whereas *P. putida* remained fairly stable (Table 4.4). Among the three bacterial strains studied, *A. calcoaceticus* was the most sensitive strain to freeze-drying, and *E. coli* was the most sensitive to subsequent storage after freeze-drying, whereas *P. putida* was the most resistant strain to both freeze-drying and subsequent storage (Figure 4.3). This result is in contrast to a previous study, in which *P. putida* was reported to be more sensitive to freeze-drying and subsequent storage than *E. coli* (Miyamoto-Shinohara et al. 2006). Based on the results presented in chapter 4, *P. putida* seemed to have the better shelf-life in the SciTox^{TM} toxicity assay than *A. calcoaceticus* and *E. coli*, irrespective of cryoprotectants and storage temperatures.

The efficiency of two cryoprotectants (PEG and sucrose/Tween 80) was evaluated based on the rate of cell viability loss and EC_{50} values to standard toxicants. The PEG pre-treated freeze-dried cells were more susceptible to two standard toxciants than their freshly cultured counterparts (Table 4.1). This might due to cell membrane damage which may have increased the cell permeability (Steponkus 1984). In contrast, sucrose/Tween 80 pre-treated freeze-dried cells were more resistant to the two

toxicants based on relatively higher EC_{50} values than their freshly cultured counterparts. The reason for this is not clear. However, sugars, including glucose and sucrose, can act as nutrients for bacteria and thereby increase cell respiratory activity (Hayek and Tipton 1966; Pasco et al. 2008). In contrast to the higher EC_{50} produced by sucrose/Tween 80, PEG seemed to be a more suitable cryoprotectant for use in the SciTox^{TM} DTA assay based on the EC_{50} values which were similar to the EC_{50}s produced by freshly cultured counterparts. However, sucrose/Tween 80 was more suitable for maintaining the viability of freeze-dried cells because these cells exhibited a significantly higher cell viability than the PEG pre-treated cells (Figure 4.3). Similar results have been reported by others (Gayle et al. 2006; Kuleshova et al. 1999). The higher cell viability observed with sucrose/Tween 80 might due to its relatively less or even no toxic effects on cells, in comparison to those treated with PEG (Gayle et al. 2006; Kuleshova et al. 1999).

In this research, the shelf-life of freeze-dried cells was also studied at two different storage temperatures (4 ˚C and -20 ˚C) under normal laboratory conditions (in a desiccator at normal atmospheric pressure) that could be easily replicated in a research or a commercial laboratory. Irrespective of the cryoprotectant, all three freeze-dried cells showed a similar dose-dependent response to the two toxciants stored at -20 ˚C for up to 3 months, compared to their freshly cultured and zero-time freeze-dried counterparts. In contrast, after 3-month stored at 4 ˚C, PEG pre-treated *A. calcoaceticus* and *E. coli* could not be used to assay toxicity of two standard toxicants in SciTox^{TM} assay (Table 4.4) because of the marked decrease in cell viability (Figure 4.3). Thus cells stored at -20 ˚C exhibited a longer shelf-life than their 4 ˚C stored counterparts. This result is similar to a previous study (Champagne et al. 1996). All three freeze-dried bacterial strains gradually lost their viability during storage at both 4 ˚C or -20 ˚C and might due to oxidation of lipids (Castro et al. 1997).

Thus it appears that all three bacterial strains pre-treated with PEG could be stored at -20 ˚C for 3 months and used to assay toxicity of chemicals in SciTox^{TM} DTA assay with results comparable to those obtained with freshly prepared cells. To my knowledge, this is the first report on evaluation of shelf-life of freeze-dried bacterial cells used in a commercial toxicity assay.

Many commercial toxicity assays use respiration as an index in toxicity determination or to monitor other metabolic events (dos Santos et al. 2002; Hansen and Sorensen 2001), including SciTox^{TM} DTA assay (Tizzard et al. 2004). Such toxicity assays are sensitive to a wider range of substances, such as metals, xenobiotics, salts (Beaton et al. 1999; Chaudri et al. 1999). However, a major disadvantage of such commercial assays is that they measure toxicity non-specifically. Any condition which decreases the metabolic activity of the biosensor bacteria, will reduce cellular respiration (Hansen and Sorensen 2001). Therefore, some highly substance-specific biosensors have been developed including enzyme-based and antibody-based ones (Andreescu and Sadik 2004; Bucur et al. 2005; Khosraviani et al. 1998; Michel et al. 2006; Rubina et al. 2005). However, such biosensors have some limitations. For example, the limited interaction between environmental compounds and enzymes and lack of specificity in differentiating among compounds of similar class is the biggest challenge for enzyme-based biosensors (Rogers 2006). The limitations of the antibody-based biosensors are the complexity of assay formats and limited compounds detected in any individual assay (Rogers 2006).

Whole-cell based biosensors offer several advantages over enzyme based sensors, including low cost, high stability and high adaptability. Recently, some substance-specific whole-cell biosensors have been developed by exploiting natural endogenous pathways (e.g. specific chemical degradation pathways) (Mulchandani et al. 2005; Tizzard et al. 2006). Furthermore, current advances in DNA technologies and genetic manipulation have improved microbial genetic information, and facilitated the development of highly specific and sensitive microbial biosensors (Hansen and Sorensen 2000; Kohlmeier et al. 2007; Li et al. 2008; Nivens et al. 2003). This type of a biosensor is genetically engineered by fusing an inducible promoter to a reporter gene.

In the second part of my research, the emphasis was to develop a genetically engineered amperometric whole-cell biosensor which used respiration as an index to detect toxicants by the SciTox^{TM} system. In order to create a substrate-specific and a sensitive bio-assay, the SciTox^{TM} assay was re-engineered by fusing a respiratory reporter gene to an inducible promoter. The presence of the target analyte was expected to increase the SciTox^{TM} signal by inducing the reporter gene expression.

Electron transfer through the bacterial respiratory chain to external acceptors (e.g. oxygen) is a biologically important phenomenon. My research aim was to use genetic techniques to manipulate electron transfer from whole cells to improve the performance of the SciTox^{TM} system. To do this, the discovery of a SciTox^{TM} suitable reporter gene was necessary. In order to find SciTox^{TM} suitable reporter genes, approximately 4000 single-gene knock-out mutants comprising the Keio *E. coli* collection (Baba et al. 2006) were tested for gain or loss of function in the SciTox^{TM} assay (Weld et al. 2010). Initially, knock-out genes from strains that gave the lowest SciTox^{TM} signals were selected for confirmation of their role in the SciTox^{TM} response. From these, four genes were selected for development of an amperometric biosensor strain on the basis of their involvement in different cellular pathways. These genes were: *nuoA*, *ompF* and *selA* (Figure 5.1). The *lacZ* gene was selected as the reporter gene for constructing the SciTox^{TM} biosensor, because it has been used previously in amperometric biosensor studies (Lehmann et al. 2000; Tag et al. 2007).

The *E. coli Tn10 tetA* promoter was chosen as a model system, because it was well characterized. In order to test the inducible promoter element, it was fused to a standard visual reporter gene *lacZ*. Then its function and response to Tet was characterized by using the blue-white colour assay. The results showed that the gene expression from *tetA* promoter transcription could be induced by Tet. Following this, the basic knowledge of Tet-induction mechanism was studied based on the *lacZ* reporter system. Three important factors, namely, cell growth phase, Tet-induction period, and substrate (X-gal) incubation time were determined at stationary-phase (Table 5.1), 90 mins, and less than 200 mins (Table 5.1), respectively.

In order to confirm that the specificity and sensitivity of SciTox^{TM} assays could be improved by gene induction in response to Tet, the response of unmodified *E. coli* to Tet was tested in SciTox^{TM} assay as the negative control. The toxicity of Tet should non-specifically inhibit *E. coli* respiration, resulting in lower SciTox^{TM} signals. However, the unmodified *E. coli* assay responded with an increased SciTox^{TM} signal in a dose-dependent manner to Tet at sub-inhibitory levels when stationary phase cells were used (Figure 5.2 A). Thus, stationary phase cells may not be suitable for use in genetically modified *E. coli* bioassays because of their unexpected and unexplained response to Tet. Therefore, exponential phase cells were used instead since these are

non-specific and produce an insensitive response to Tet at sub-inhibitory levels (Figure 5.2 B).

In order to construct Tet-specific SciTox^{TM} biosensors, four different strategies were used. In the first strategy, the quantity of metabolizable carbon available to the bacteria in the SciTox^{TM} assay was manipulated. However, the assay did not respond to Tet in a dose-dependent manner. The reason is not clear. It is known that the expression of *lacZ* could be induced by the presence of Tet (Table 5.1). Also *lacZ* has been used as an amperometric reporter gene to detect and quantify Cu^{2+} in yeast (Lehmann et al. 2000; Tag et al. 2007). The possible reason for this might due to the low expression of *lacZ* during the exponential growth-phase.

In the second strategy, the overall redox activity of respiratory enzymes was manipulated. To do this, *selA* was fused to the *tetA* promoter, and transferred into a Δ*selA* strain. The *selA*-based bioassay was then tested at a range of Tet concentrations. However, the assay responded to Tet in a similar dose-dependent manner as unmodified *E. coli* assay (Figure 5.2 B). It is unclear why *selA* cannot be used as a reporter gene to detect Tet sensitively in SciTox^{TM} system, as *selA* expression is known to significantly affect the SciTox^{TM} response (Weld et al. 2010). The increase in respiration rate is not directly related to the action of the SelA protein but the action of the newly synthesized seleno-proteins after the induction of *selA*. It might be that turn-over and expression of seleno-proteins may be quite low during the SciTox^{TM} assay, and the respiration rate of sensor cells might not be affected by low levels of seleno-proteins.

In the third strategy, the passive diffusion efficiency of the mediator KFCIII across the outer membrane of *E. coli* sensor cells was manipulated. The *ompF* gene encodes an outer-membrane protein ompF, which forms a large porin channel involved in passive diffusion across the outer membrane of Gram-negative bacteria, including *E. coli* (Bekhit et al. 2011; Garcia-Gimenez et al. 2011; Housden et al. 2010). The *ΔompF* pSimon assay responded to Tet in a sensitive dose-dependent manner (Figure 5.3) in comparison to unmodified *E. coli* assay (Figure 5.2 B). The DL of Tet determined by the assay was 0.013 μg ml^{-1}, which was about one order of magnitude more sensitive than the EU MRL of Tet (Virolainen et al. 2008). In contrast to the visual reporter gene based whole-cell biosensors, the DL of Tet by *ΔompF* pSimon

assay was at least as sensitive as the whole-cell biosensors (Hansen and Sorensen 2000; Virolainen et al. 2008) and very close to the DL of a more recent luminescent-based *E. coli* whole cell biosensor (Pikkemaat et al. 2010). Loss of SciTox^{TM} signal at Tet concentrations above 0.1 μg ml^{-1} (Figure 5.3 A) was not due to Tet toxicity (Figure 5.2 B) but probably due to the over-expression of the transgene leading to formation of protein inclusion bodies.

In the fourth strategy, a key respiratory gene *nuoA* was chosen as a reporter gene for use in a SciTox^{TM} amperometric biosensor. The *nuo* operon encodes enzyme NADH dehydrogenase I, which plays an important role in the cell respiratory chain pathway (Wackwitz et al. 1999, 2000). The presence of a specific analyte could be detected by a measurable increase in SciTox^{TM} signal due to the direct action of the induction of *nuoA* expression. The *ΔnuoA* pSong10 assay responded to Tet in a more sensitive dose-dependent manner (Figure 5.5) than *ΔompF* pSimon assay (Figure 5.3). The DL of Tet determined by this assay was 0.0026 μg ml^{-1} (Figure 5.5 B), which was about one order of magnitude more sensitive to Tet than visual reporter gene-based whole cell bioassays, such as *gfp*, *luxCDABE* and *lacZ* (Hansen and Sorensen 2000; Pellegrini et al. 2004; Shen et al. 2011; Virolainen et al. 2008). In contrast to other types of bioassay such as SPR-sensor assay and microbial inhibition assay, the *nuoA*-based bioassay offers higher Tet sensitivity (Moeller et al. 2007; Nagel et al. 2011). The sensitivity of Tet determined by *nuoA*-based SciTox^{TM} assay was lower than with LC-UV methods (Andersen et al. 2005; Kaale et al. 2008; Lee et al. 2007; Zhou et al. 2009) and very close to those reported in LC-MS/MS (Granelli et al. 2009; Xu et al. 2008; Yue et al. 2006) and HPLC based assays (Jing et al. 2011; Shalaby et al. 2011).

To my knowledge, this is the first time a respiratory gene has been used as a reporter gene in an amperometric biosensor, and provided high sensitivity to a specific target, Tet. The Δ*nuoA* pSong10 assay could detect Tet at the 10^{-3} to 10^{-1} μg ml^{-1} range (Figure 5.5). The SciTox^{TM} signal declined rapidly at Tet above 0.1 μg ml^{-1}. This might have been due to either Tet toxicity (Figure 5.2 B) and/or protein inclusion body formation.

To confirm that *nuoA*-based biosensor could detect other environmental compounds rather than Tet, the *nuoA* gene was fused to the *copA* promoter in plasmid pSong11.

The Δ*nuoA* pSong11 bioassay responded to either Ag or Cu in a dose-dependent manner (Figure 5.6).

Two aspects of the genetically modified bioassays need to be improved. Firstly, loss of SciTox^{TM} signals at high concentrations of Tet (Figure 5.3 and 5.5) reduces the effectiveness of these assays. This limitation might be caused by either Tet toxicity and/or transgene over-expression. Secondly, a relatively high background current at zero analyte recorded in genetically modified *E. coli* bioassays, lowers the DL of the analyte. This limitation could be caused by the transgene basal expression due to the *tetA* promoter being incompletely turned off. Therefore, the DL and optimal induction of SciTox^{TM} bioassays could be improved by overcoming these two limitations. The *nuoA*-based bioassay was used as a model system because it has a high sensitivity compared to the *ompF*-based bioassay.

In order to overcome Tet toxicity (Figure 6.1), and thereby improving the SciTox^{TM} response at high Tet concentration (optimal Tet induction), the f' plasmid, encoding Tet resistance, was transferred into the sensor cells. However, Tet-resistance did not improve the assay on its own (Figure 6.2). Transgene over-expression was also speculated to play a role in decline of the SciTox^{TM} signal at high Tet concentrations. Two strategies were used to overcome the gene over-expression. In the first strategy, a low induction temperature (15 ˚C) was used to reduce the protein synthesis rate thereby reducing inclusion bodies (Lethanh et al. 2005). In the second strategy, copy number of the transgene was reduced by using a low-copy number plasmid (pBR322) as the vector to carry the transgene instead of pBluescript. Neither strategy improved the response of *nuoA* assay to Tet (Figure 6.5).

However, by combining the strategies to reduce gene over-expression with Tet-resistance, the SciTox^{TM} response at high Tet concentrations was significantly improved and the optimal Tet induction concentration was improved up to 1 μg ml^{-1} (Figure 6.3 and 6.4). Despite this, loss of SciTox^{TM} signal was still observed at Tet concentrations below inhibitory levels (1 – 10 μg ml^{-1}) (Figure 6.3 and 6.4). Additionally, by using a low-copy number plasmid, the background expression of the transgene was significantly reduced and the DL of Tet was improved to 0.00023 μg ml^{-1} (Figure 6.6). Even with analyte resistance and low-copy number of the transgene, it was still not possible to prevent the assay signal from declining below the

background level at high analyte concentrations (Figure 6.3 and 6.4) and the background expression from being higher than that of the non-transgenic control (Figure 6.6).

7.2 Problems and Future Research

It was expected that the specificity and sensitivity of the SciTox^{TM} assay could be improved by using the genetically engineered whole-cell based biosensors. However, the genetically engineered whole-cell biosensors have some inherent advantages, problems and limitations. Firstly, the recombinant protein expression system used in this research was *E. coli*, which has many advantages including that it is easy and cheap to grow with a high yield of proteins. However, the formation of protein inclusion bodies is a common limitation of recombinant protein production in bacteria (Ventura and Villaverde 2006), especially in *E. coli* (Mayer and Buchner 2004).

There are a number of methods that could be used alternatively to decrease the formation of inclusion bodies (Cantrell 2003; Mayer and Buchner 2004; Strandberg and Enfors 1991) and thereby improve the response of bioassays to the target analyte. For example, reducing the inducible promoter strength by reducing the identity of either hexamers located at positions of -35 (TTGACA) and -10 (TATAAT) (Babb et al. 2004) and/or SDS (AGGAGGT) located at 6-8 bps upstream of the start codon (Shine and Dalgarno 1974) could significantly reduce the transcription efficiency of the transgene (McCracken and Timms 1999; Starmer et al. 2006). Over-expression of chaperone proteins (e.g. DnaK/DnaJ) along with the reporter gene could be used to increase the folding efficiency of the target protein (Chen et al. 2003), thereby preventing aggregation. Expression of heat shock proteins (e.g. Hsp70/IbpA/IbpB) in the sensor cells might be used to avoid inactivation and aggregation of the target proteins and also to facilitate their subsequent refolding by chaperones (Jurgen et al. 2010). Integration of the entire transgene into the chromosome could further reduce copy-number down to 1 copy/chromosome (Alonso et al. 2003). A low recombinant protein expression system (e.g. yeast) might be useful to reduce the yields of protein products, instead of *E. coli* (Cantrell 2003).

Secondly, even with a low-copy number plasmid, the background SciTox™ signal from uninduced transgenic cells was still significantly higher than the background SciTox™ signal obtained from the parent strain without the inducible transgene construct. It indicated that the amount of regulatory protein (TetR) produced was not sufficient to block the transcription from the inducible promoter (*tetA*). Several methods might improve TetR regulation. Mutations in the sequence of operators could reduce binding affinity between TetR and the operators, thereby up-regulating the transcription of TetR from *tetR* promoters. Increasing the *tetR* promoters' strength by mutation could increase the transcription efficiency of TetR from the promoters. Additionally, some methods used to overcome gene over-expression might also be used to reduce gene basal expression. For example, decrease of inducible promoter strength could reduce the expression efficiency of the reporter gene and the copy-number of the transgene could be further reduced by integrating the transgene into a chromosome.

Thirdly, genetically modified SciTox™ assay might not distinguish the compounds within the same class. This is probably caused by the limitations of substrate-inducible promoters. The inducible promoter could be induced by compounds that have similar structures. For example, the *Tn10 tetA* promoter (from *E. coli*) has been reported to be induced by Tet and its epimers (chlortetracycline, doxycycline and oxytet) (Virolainen et al. 2008).

Fourthly, the genetically engineered whole cell biosensors are unstable. For example, all cells of sensors used in SciTox™ assay had to be produced fresh each day. If the cells were left in the refrigerator at 4 ℃ for more than 24 hours, the performance of the bioassay declined (the relative SciTox™ peak value was significantly lower), and sometimes did not even respond at all. The genetically engineered bacterial sensor had to be re-created (re-transformed into *E. coli* hosts) after 6 months storage at -80 ℃, because of loss of response in the SciTox™ toxicity assay.

Finally, the genetically modified biosensors that I developed were only tested using samples in distilled water and in milk, and it is not known how these would respond to environmental contaminant mixtures. Overall, the genetically engineered whole-cell amperometric biosensor offered high sensitivity and specificity to specific targets. However, this technology is still at an infancy stage.

In future research, it would be useful to improve the speed of the SciTox^{TM} assay and to evaluate the effects of using samples in more complex matrices such as environmental and industrial samples before it is used in other real-world applications. Strategies need to be developed to minimize inclusion body formation during gene induction. The entire dose-response curve and linear dose-response range could be improved by reducing the formation of inclusion bodies. Furthermore, the research could be facilitated by combining the specific SciTox^{TM} whole cell biosensors together with the freeze-dried technology. Biosensor cells may reduce or arrest their mutations when freeze-dried. So the freeze-drying may improve the stability of biosensor cells in SciTox^{TM} assay.

CHAPTER 9: REFERENCES

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CHARPTER 10: APPENDICES

Appendix 1. Restriction Endonucleases related React® buffers

Table 10.1. Compositions of different React® buffers (10 × React® buffer is normally used with every Restriction enzyme. The buffer compositions shown below are at 1 × concentration).

Type of React® buffers	Compositions
React® 1	50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$
React® 2	50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl
React® 3	50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl
React® 4	20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM KCl
React® 5	10 mM Tris-HCl (pH 8.2), 8 mM $MgCl_2$
React® 6	50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl
React® 7	50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl
React® 8	20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$
React® 9	20 mM Tris-OAc (pH 7.9), 10 mM MgOAc, 50 mM KOAc
React® 10	100 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 150 mM NaCl
React® 11	10 mM Tris-HCl (pH 9.0), 12 mM $MgCl_2$, 100 mM KCl

Appendix 2. Vector gene sequences

Table 10.2. The pBluescript (-) gene sequences

pBluescript SK(-), 2958 bp version 122001

Note: The following sequence has been verified for accuracy at the junctions. The remainder of the sequence has been obtained from existing data.

```
   1 CACCTGACGC GCCCTGTAGC GGCGCATTAA GCGCGGCGGG TGTGGTGGTT
  51 ACGCGCAGCG TGACCGCTAC ACTTGCCAGC GCCCTAGCGC CCGCTCCTTT
 101 CGCTTTCTTC CCTTCCTTTC TCGCCACGTT CGCCGGCTTT CCCCGTCAAG
 151 CTCTAAATCG GGGGCTCCCT TTAGGGTTCC GATTTAGTGC TTTACGGCAC
 201 CTCGACCCCA AAAAACTTGA TTAGGGTGAT GGTTCACGTA GTGGGCCATC
 251 GCCCTGATAG ACGGTTTTTC GCCCTTTGAC GTTGGAGTCC ACGTTCTTTA
 301 ATAGTGGACT CTTGTTCCAA ACTGGAACAA CACTCAACCC TATCTCGGTC
 351 TATTCTTTTG ATTTATAAGG GATTTTGCCG ATTTCGGCCT ATTGGTTAAA
 401 AAATGAGCTG ATTTAACAAA AATTTAACGC GAATTTTAAC AAAATATTAA
 451 CGCTTACAAT TTCCATTCGC CATTCAGGCT GCGCAACTGT TGGGAAGGGC
 501 GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA AGGGGGATGT
 551 GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTTCCC AGTCACGACG
 601 TTGTAAAACG ACGGCCAGTG AATTGTAATA CGACTCACTA TAGGGCGAAT
 651 TGGGTACCGG GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGATATC
 701 GAATTCCTGC AGCCCGGGGG ATCCACTAGT TCTAGAGCGG CCGCCACCGC
 751 GGTGGAGCTC CAGCTTTTGT TCCCTTTAGT GAGGGTTAAT TTCGAGCTTG
 801 GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC
 851 AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGG
 901 CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT
 951 TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG
1001 CGCGGGGAGA GGCGGTTTGC GTATTGGGCG CTCTTCCGCT TCCTCGCTC
1051 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCA
1101 TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
1151 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG
1201 CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
1251 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
1301 CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
1351 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG
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1401 CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
1451 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG
1501 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
1551 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT
1601 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
1651 GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA
1701 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
1751 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
1801 AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
1851 AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC
1901 CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
1951 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
2001 ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT
2051 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG
2101 CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA
2151 AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC
2201 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT
2251 CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG
2301 GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG
2351 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT
2401 CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
2451 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT
2501 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT
2551 AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT ACGGGATAAT
2601 ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
2651 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA
2701 TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC
2751 AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG
2801 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT
2851 ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT
2901 GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
2951 AAAAGTGC

Appendix 3. DNA sequences of inducible-promoters and reporter genes

Table 10.3. *TetA* promoter and *tetR* regulatory gene

CTATGATTCC CTTTGTCAAC AGCAATGGAT CACTGAAAAT GGTTCAATGA TCACATTAAG
TGGTATTCAA TATTTTCATG AAATGGGAAT TGACGTTCCT TCCAAACATT CACGTAAAAT
CTGTTGTGCG TGTTTAGATT GGAGTGAACG CCGTTTCCAT TTAGGTGGGT ACGTTGGAGC
CGCATTATTT TCGCTTTATG AATCTAAAGG GTGGTTAACT CGACATCTTG GTTACCGTGA
AGTTACCATC ACGGAAAAAG GTTATGCTGC TTTTAAGACC CACTTTCACA TTTAAGTTGT
TTTTCTAATC CGCATATGAT CAATTCAAGG CCGAATAAGA AGGCTGGCTC TGCACCTTGG
TGATCAAATA ATTCGATAGC TTGTCGTAAT AATGGCGGCA TACTATCAGT AGTAGGTGTT
TCCCTTTCTT CTTTAGCGAC TTGATGCTCT TGATCTTCCA ATACGCAACC TAAAGTAAAA
TGCCCCACAG CGCTGAGTGC ATATAATGCA TTCTCTAGTG AAAAACCTTG TTGGCATAAA
AAGGCTAATT GATTTTCGAG AGTTTCATAC TGTTTTTCTG TAGGCCGTGT ACCTAAATGT
ACTTTTGCTC CATCGCGATG ACTTAGTAAA GCACATCTAA AACTTTTAGC GTTATTACGT
AAAAAATCTT GCCAGCTTTC CCCTTCTAAA GGGCAAAAGT GAGTATGGTG CCTATCTAAC
ATCTCAATGG CTAAGGCGTC GAGCAAAGCC CGCTTATTTT TTACATGCCA ATACAATGTA
GGCTGCTCTA CACCTAGCTT CTGGGCGAGT TTACGGGTTG TTAAACCTTC GATTCCGACC
TCATTAAGCA GCTCTAATGC GCTGTTAATC ACTTTACTTT TATCTAATCT AGACATCATT
AATTCCTAAT TTTTGTTGAC ACTCTATCAT TGATAGAGTT ATTTTACCAC TCCCTATCAG
TGATAGAGAA AAGTGAAATG

Table 10.4. *CopA* promoter gene sequences

ATTTTGTCCGCCGTTAAGTGAGTGAAATTGGGTGTAAGCCTGATCCACTGCCTGCTG
TAATTTGTTTGCATCTAACATCTTTTGTTAACTCCTTTTTATAGATGCGGGAGGTAA
TTCCTCACCCCGGTGCCGATTTTCAGGCATCCTGATTTAACTTAGCACCCGCAACTT
AACTACAGGAAAACAAAGAGATAAATGTCTAATCCTGATGCAAATCGAGCCGATTTT
TTAATCTTTACGGACTTTTACCCGCCTGGTTTATTAATTTCTTGACCTTCCCCTTGC
TGGAAGGTTTAACCTTTATCACAGCCAGTCAAAACTGTCTTAAAGGAGTGTTTTATG

Table 10.5. DNA sequences of *selAB* gene

```
2581 atgacaaccg aaacgcgctc cctctatagt caacttccgg ctattgatcg cttattgcgc
2641 gatagctcct tcctttcttt gcgtgatact tatggtcaca cccgcgtggt ggaattgttg
2701 cgtcagatgc tcgacgaagc gcgagaagtg attcgtggca gccagacgct gcctgcgtgg
2761 tgtgaaaact gggcgcaaga agtcgatgcc cggttgacga aagaagcgca gagcgcgctg
2821 cgtccggtga tcaacctgac gggaaccgtg ctgcatacca accttgggcg agctttacag
2881 gcggaagccg cggtggaagc cgttgcgcag gctatgcgtt cgccagtgac cctcgagtat
2941 gatctggacg acgccggacg cggacatcgc gatcgggcgc tggcgcagct gctgtgccgt
3001 attacggggg cggaagatgc ctgtatcgtc aataacaatg cggcggcggt gttattgatg
3061 ttggcggcca ctgccagcgg aaaagaggtg gtggtatctc gcggcgaact ggtggagatt
3121 ggcggcgcgt ttcgtattcc cgatgttatg cgtcaggcag gctgcaccct acacgaagta
3181 gggaccacca accgcacgca cgcgaatgat tatcgtcagg cggtgaatga aaataccgca
3241 ctgttgatga aagtacatac cagtaactac agcattcagg ggttcaccaa agcgatagat
3301 gaagcggaac tggtggcgct cggcaaagag ctggatgttc ccgtagtgac tgatttaggc
3361 agtggctcgc tggtcgatct tagccagtac ggtttgccga aagagccaat gccgcaggag
3421 ttgattgcgg cgggcgtcag tctggtgagt ttctccggcg acaagttgtt aggcgggccg
3481 caggcaggaa ttattgttgg taaaaaagag atgatcgccc gcctgcaaag ccacccgctg
3541 aagcgtgcat tacgcgcgga taaaatgacc ctcgcggcgc tggaagccac gttgcgtctt
3601 tatttacacc ctgaagctct gagtgaaaaa ttaccgaccc tgcgcctgct tacccgcagc
3661 gcagaggtca ttcaaatcca ggcacaacgt ttacaggccc cccttgccgc acattacggc
3721 gcggagtttg cggtacaggt tatgccatgt ctttcgcaga ttggcagtgg ttcgctgccg
3781 gttgatcgcc tgccgagcgc ggcattaacg tttacacccc atgatggacg cggtagccac
3841 cttgagtcat tagccgcccg ctggcgtgaa ttgccagtgc cggtgattgg tcgtatttat
3901 gacggacgat tgtggctgga tttacgctgc cttgaagatg agcaacggtt tttggagatg
3961 ttgttgaaat gattattgcg actgccggac acgttgacca cggcaaaaca accttattgc
4021 aggcgattac tggcgtaaat gctgaccgtc tgccggaaga aaaaaagcgc ggcatgacca
4081 tcgatctcgg ctatgcctac tggccgcagc cggatggtcg cgtgcctggt tttatcgacg
4141 ttcccggtca tgaaaagttt ctttccaaca tgctggcggg cgttggtggt atcgatcacg
4201 cgctgttggt ggtggcgtgc gatgacggcg tgatggcaca gacccgtgag catctggcga
4261 ttttgcagct gaccggtaac ccgatgctga cagtggcgct gaccaaagcc gatcgcgtgg
4321 acgaagcgcg tgttgatgag gttgaacgcc aggtaaagga ggttctgcgg gaatacggtt
4381 ttgctgaggc aaaactgttt atcaccgcag caaccgaagg tcggggaatg gatgccctgc
4441 gcgagcatct gcttcagttg ccggaacgcg agcacgccag ccaacatagt ttccgcctcg
4501 cgattgaccg cgcatttacc gtaaaaggtg ccgggctggt cgtcaccggt acggcgttaa
4561 gcggggaagt gaaggtaggc gattcactct ggctgactgg tgtaaataaa ccgatgcgtg
4621 tacgtgcgct gcatgcgcaa aaccagccaa cagaaaccgc caatgccggg cagcgtatcg
4681 cgcttaacat cgcgggtgat gcggaaaaag agcagattaa ccgtggcgac tggctgcttg
4741 ccgatgtgcc gccagagccg ttcacacggg tgattgtcga gcttcaaacc catacaccgc
4801 tgacccagtg gcagccgctg catattcacc acgccgccag ccacgtcacg ggacgcgttt
4861 cactgctgga agataacctt gctgaactgg tcttcgacac cccgttatgg ctggcagata
4921 acgaccgcct ggtattgcgc gatatctctg cccgcaacac gctggccgga gcgcgcgtcg
4981 tgatgcttaa cccgccgcgt cgcggtaaac gtaagccgga atatctgcaa tggctggcgt
5041 ctcttgcacg ggcgcagagc gatgccgatg cgttatctgt tcatctggaa cgcggcgcgg
5101 ttaaccttgc ggatttcgcc tgggcgcgcc agctcaacgg cgaagggatg cgcgaattgc
5161 tgcaacagcc tggttatatt caggctggtt atagcttgtt gaatgcgccg gttgccgccc
5221 gctggcagcg gaaaattctc gacacattag cgacttatca tgagcaacat cgcgatgaac
5281 ctggccctgg gcgcgaacgt ctgcgacgta tggcgttgcc aatggaagat gaagcgctgg
5341 tactgttgct gattgaaaag atgcgcgaaa gcggcgacat ccacagccat cacggctggc
5401 tgcatctgcc agatcacaaa gcgggcttca gcgaagagca gcaggccatc tggcaaaaag
5461 cagagccact gtttggtgac gaaccgtggt gggtgcgtga cctggcaaaa gagacgggaa
5521 ccgacgagca ggcaatgcgc ctgactctac gccaggcggc gcagcaagga ataattaccg
5581 cgatcgttaa agatcgttat taccgtaacg atcggattgt cgagtttgcc aatatgatcc
5641 gcgatctcga tcaggagtgt ggttcaacct gcgcggcgga tttccgcgat cgcttaggcg
5701 taggccgaaa gctggcaatt cagattctgg aatattttga ccgcattggc ttt
```

Table 10.6. DNA sequences of *nuoA* gene

```
gcatgtacgc ttcaggacgc ggcgggcagc ccgggatata cacatcaacc
gggatgaatt tatcgacgcc ctgcacaacg gaataaatat cgtacatacc
accagagttg gcacaggcac ccattgagat aacccatttt ggttccagca
tctggtcata cagacgctga ataaccggtg ccattttggt aaagcaggtt
cctgcaacca ccatcaggtc agcctgacgc ggcgaagcac gcaatacttc
tgcgccaaaa cgcgccacgt catgcaccgc ggtaaacgaa gtcaccatct
caacgtaaca gcaggaaaga ccgaagttat acggccaaat tgagttttta
cgaccccagt taaccatgtc attgagcttg cccataaaca cgtttttgtt
aacttcttgc tccagagggt cggttacgat ctcctgcttt tgcagggggt
aacggtcgtt ctcaccgttg ggatctatgc gggtgagcgt ataatccatc
ttaatgcctc gcggttagcg ttgacgatta gcgatactgt tcgtttccgg
gttcatacgc tcgcggcgtg aacgcgcggg cgtccagtcc agcgcgccaa
tacgcaccag ataaaccaga cctgccagta acacaaaaat aaaaattgca
gcttccacaa agcctaccca gccgctttcg cggatagagg ttgaccatgc
gaacagatac agcgcttcaa cgtcgaagat aacgaagaac atggccacca
gataaaactt ggcggacagg cgtaagcggg cggagccgac cgagtcgata
ccggattcaa acggcacgtt tttcgacctc gcgcgtgcgc gaccgcctaa
aaaccaaccg cctaccagca tcaggcaaca caggccaatg gcaacgataa
gaaagatagc gaatgcccag tgatgagcga tgacttcagt ggatgttgac
atactcat
```

Table 10.7. DNA sequences of *ompF* gene

```
catgagggta  ataaataatg  atgaagcgca  atattctggc  agtgatcgtc
cctgctctgt  tagtagcagg  tactgcaaac  gctgcagaaa  tctataacaa
agatggcaac  aaagtagatc  tgtacggtaa  agctgttggt  ctgcattatt
tttccaaggg  taacggtgaa  aacagttacg  gtggcaatgg  cgacatgacc
tatgcccgtc  ttggttttaa  aggggaaact  caaatcaatt  ccgatctgac
cggttatggt  cagtgggaat  ataacttcca  gggtaacaac  tctgaaggcg
ctgacgctca  aactggtaac  aaaacgcgtc  tggcattcgc  gggtcttaaa
tacgctgacg  ttggttcttt  cgattacggc  cgtaactacg  gtgtggttta
tgatgcactg  ggttacaccg  atatgctgcc  agaatttggt  ggtgatactg
catacagcga  tgacttcttc  gttggtcgtg  ttggcggcgt  tgctacctat
cgtaactcca  acttctttgg  tctggttgat  ggcctgaact  tcgctgttca
gtacctgggt  aaaaacgagc  gtgacactgc  acgccgttct  aacggcgacg
gtgttggcgg  ttctatcagc  tacgaatacg  aaggctttgg  tatcgttggt
gcttatggtg  cagctgaccg  taccaacctg  caagaagctc  aacctcttgg
caacggtaaa  aaagctgaac  agtgggctac  tggtctgaag  tacgacgcga
acaacatcta  cctggcagcg  aactacggtg  aaacccgtaa  cgctacgccg
atcactaata
```

Appendix 4. Plasmid Sequencing Results

Table 10.8. pSong8 sequencing results

```
PSong8   12   AGCGCCC-ATACGC-AACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGC  69

              ||||||| |||||| ||||||||||||||||||||||||||||||||||||||||||||
PBlues   102  AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGC  43
                                                PstI
PSong8   70   ACGACAGGTTTCCCGACTGGAAAGCGGGCAGTCTGCAG  107
              |||||||||||||||||||||||||||||||||
PBlues   42   ACGACAGGTTTCCCGACTGGAAAGCGGGCAGT------  11

PSong8   108  AATGGGAATTGACGTTCCTTCCAAACATTCACGTAAAATCTGTTGTGCGTGTTTAGATTG  167
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   82   AATGGGAATTGACGTTCCTTCCAAACATTCACGTAAAATCTGTTGTGCGTGTTTAGATTG  141

PSong8   168  GAGTGAACGCCGTTTCCATTTAGGTGGGTACGTTGGAGCCGCATTATTTTCGCTTTATGA  227
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   142  GAGTGAACGCCGTTTCCATTTAGGTGGGTACGTTGGAGCCGCATTATTTTCGCTTTATGA  201

PSong8   228  ATCTAAAGGGTGGTTAACTCGACATCTTGGTTACCGTGAAGTTACCATCACGGAAAAAGG  287
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   202  ATCTAAAGGGTGGTTAACTCGACATCTTGGTTACCGTGAAGTTACCATCACGGAAAAAGG  261

PSong8   288  TTATGCTGCTTTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGCATATGATC  347
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   262  TTATGCTGCTTTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGCATATGATC  321

PSong8   348  AATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGCT  407
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   322  AATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGCT  381

PSong8   408  TGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTCTTCTTTAGCGACT  467
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   382  TGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTCTTCTTTAGCGACT  441

PSong8   468  TGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAATGCCCCACAGCGCTGAGTGCA  527
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   442  TGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAATGCCCCACAGCGCTGAGTGCA  501

PSong8   528  TATAATGCATTCTCTAGTGAAAAACCTTGTTGGCATAAAAAGGCTAATTGATTTTCGAGA  587
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   502  TATAATGCATTCTCTAGTGAAAAACCTTGTTGGCATAAAAAGGCTAATTGATTTTCGAGA  561

PSong8   588  GTTTCATACTGTTTTTCTGTAGGCCGTGTACCTAAATGTACTTTTGCTCCATCGCGATGA  647
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   562  GTTTCATACTGTTTTTCTGTAGGCCGTGTACCTAAATGTACTTTTGCTCCATCGCGATGA  621

PSong8   648  CTTAGTAAAGCACATCTAAAACTTTTAGCGTTATTACGTAAAAAATCTTGCCAGCTTTCC  707
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   622  CTTAGTAAAGCACATCTAAAACTTTTAGCGTTATTACGTAAAAAATCTTGCCAGCTTTCC  681

PSong8   708  CCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTATCTAACATCTCAATGGCTAAGGCGTCG  767
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   682  CCTTCTAAAGGGCAAAAGTGAGTATGGTGCCTATCTAACATCTCAATGGCTAAGGCGTCG  741

PSong8   768  AGCAAAGCCCGCTTATTTTTTACATGCCAATACAATGTAGGCTGCTCTACACCTAGCTTC  827
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   742  AGCAAAGCCCGCTTATTTTTTACATGCCAATACAATGTAGGCTGCTCTACACCTAGCTTC  801

PSong8   828  TGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCTCATTAAGCAGCTCTAATGCG  887
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro   802  TGGGCGAGTTTACGGGTTGTTAAACCTTCGATTCCGACCTCATTAAGCAGCTCTAATGCG  861

PSong8   888  CTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTAATTCCTAATTTTTTGTTGAC  947
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
```

```
Tetpro  862  CTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTAATTCCTAATTTTT-GTTGAC  920

                                                          SDS    HindIII
PSong8  948  ACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGAAAAAAAGCTT   1006
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PBlues  921  ACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGAAAA-------   972

PSong8  1007  ATGACCATGATTACGCCCAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAAGCTGG  1064
              |||||||||||| |||||||||||||||||||||||||||||||||| |||||
PBlues  153   ATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAA-GCTGG  110
```

Table 10.9. pSong9 Sequencing Results

Direction: Forward

```
Psong9  5    GCAGCTCTAATGCGCTGTT-ATCACTTTACTTTTATCTAATCTAGACATCATTAATTCCT  63
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Tetpro  848  GCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTAATTCCT  907

SDS
PSong9  64   AATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGA  123
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro  908  AATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGA  967
                 HindIII
PSong9  124  GAAAAAGGCTT  134
             |||||
Tetpro  968  GAAAA------  972

PSong9  135   ATGACAACCGAAACGCGTTCCCTCTATAGTCAACTTCCGGCTATTGATCGCTTATTGCGC  194
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  1       ATGACAACCGAAACGCGTTCCCTCTATAGTCAACTTCCGGCTATTGATCGCTTATTGCGC  60

PSong9  195   GATAGCTCCTTCCTTTCTTTGCGTGATACTTATGGTCACACCCGCGTGGTGGAATTGTTG  254
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  61      GATAGCTCCTTCCTTTCTTTGCGTGATACTTATGGTCACACCCGCGTGGTGGAATTGTTG  120

PSong9  255   CGTCAGATGCTCGACGAAGCGCGAGAAGTGATTCGTGGCAGCCAGACGCTGCCTGCGTGG  314
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  121     CGTCAGATGCTCGACGAAGCGCGAGAAGTGATTCGTGGCAGCCAGACGCTGCCTGCGTGG  180

PSong9  315   TGTGAAAACTGGGCGCAAGAAGTCGATGCCCGGTTGACGAAAGAAGCGCAGAGCGCGCTG  374
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  181     TGTGAAAACTGGGCGCAAGAAGTCGATGCCCGGTTGACGAAAGAAGCGCAGAGCGCGCTG  240

PSong9  375   CGTCCGGTGATCAACCTGACGGGAACCGTGCTGCATACCAACCTTGGGCGAGCTTTACAG  434
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  241     CGTCCGGTGATCAACCTGACGGGAACCGTGCTGCATACCAACCTTGGGCGAGCTTTACAG  300

PSong9  435   GCGGAAGCCGCGGTGGAAGCCGTTGCGCAGGCTATGCGTTCGCCAGTGACCCTCGAGTAT  494
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  301     GCGGAAGCCGCGGTGGAAGCCGTTGCGCAGGCTATGCGTTCGCCAGTGACCCTCGAGTAT  360

PSong9  495   GATCTGGACGACGCCGGACGCGGACATCGCGATCGGGCGCTGGCGCAGCTGCTGTGCCGT  554
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  361     GATCTGGACGACGCCGGACGCGGACATCGCGATCGGGCGCTGGCGCAGCTGCTGTGCCGT  420

PSong9  555   ATTACGGGGGCGGAAGATGCCTGTATCGTCAATAACAATGCGGCGGCGGTGTTATTGATG  614
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  421     ATTACGGGGGCGGAAGATGCCTGTATCGTCAATAACAATGCGGCGGCGGTGTTATTGATG  480

PSong9  615   TTGGCGGCCACTGCCAGCGGAAAAGAGGTGGTGGTATCTCGCGGCGAACTGGTGGAGATT  674
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  481     TTGGCGGCCACTGCCAGCGGAAAAGAGGTGGTGGTATCTCGCGGCGAACTGGTGGAGATT  540

PSong9  675   GGCGGCGCGTTTCGTATTCCCGATGTTATGCGTCAGGCAGGCTGCACCCTACACGAAGTA  734
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  541     GGCGGCGCGTTTCGTATTCCCGATGTTATGCGTCAGGCAGGCTGCACCCTACACGAAGTA  600
```

```
PSong9  735   GGGACCACCAACCGCACGCACGCGAATGATTATCGTCAGGCGGTGAATGAAAATACCGCA  794
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  601     GGGACCACCAACCGCACGCACGCGAATGATTATCGTCAGGCGGTGAATGAAAATACCGCA  660

PSong9  795   CTGTTGATGAAAGTACATACCAGTAACTACAGCATTCAGGGGTTCACCAAAGCGATAGAT  854
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  661     CTGTTGATGAAAGTACATACCAGTAACTACAGCATTCAGGGGTTCACCAAAGCGATAGAT  720

PSong9  855   GAAGCGGAACTGGTGGCGCTCGGCAAAGAGCTGGATGTTCCCGTAGTGACTGATTTAGGC  914
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  721     GAAGCGGAACTGGTGGCGCTCGGCAAAGAGCTGGATGTTCCCGTAGTGACTGATTTAGGC  780

PSong9  915   AGTGGCTCGCTGGTCGATCTTAGCCAGTACGGTTTGCCGAAAGAGCCAATGCCGCAG-AG  973
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
SelA  781     AGTGGCTCGCTGGTCGATCTTAGCCAGTACGGTTTGCCGAAAGAGCCAATGCCGCAGGAG  840

PSong9  974   TTTGATTGCGGGCGGGCGTCAGTCTGGGTGAGTTTCTCCGGGCGACAAGTTTGTTTAG-C  1032
              || |||||||| ||||||||||||||| |||||||||||| ||||||||| ||| || |
SelA  841     TT-GATTGCGG-CGGGCGTCAGTCTGG-TGAGTTTCTCCGG-CGACAAGTT-GTT-AGGC  894

PSong9  1033  GGGGCGGCAG-CAGGTAATTTATTTGTTGTTAAAAAAAGAGATTGATCGCCCGCCTTGCC  1091
              ||| || ||| |||| |||| ||| |||| ||||||| ||||| ||||||||||||| ||
SelA  895     GGGCCG-CAGGCAGG-AATT-ATT-GTTGGTAAAAAA-GAGAT-GATCGCCCGCCT-GCA  947

PSong9  1092  AAGCCAACCCGGCTGGAAGGCGCTGCATTACGCGCGGATTAAATGGACCCT  1142
              |||||| |||| ||| ||| || ||||||||||||||| ||||| |||||
SelA  948     AAGCCA-CCCG-CTG-AAG-CG-TGCATTACGCGCGGATAAAATG-ACCCT  992
```

Direction: Backward

No more restriction site here (re-ligated by PCR products with SalI/XhoI digested)

```
PSong9  33    GTCCACCAGAGATATTCGCGCATTACCACGCGGTCGTTATCTGCCAGGCATAA-GGTTGAAAGAAC  97
                     |||||||| ||||||| ||||| |||||||||||||||||| ||||| ||  |   |  |
SelA  2370    ------CAGAGATA-TCGCGCAATACCAGGCGGTCGTTATCTGCCAGCCATAACGG--G-GTG-TC  2316

PSong9  98    AGAAAAAGACCGGTTCAGCAAGGTTATCTTCCAGCAGTGAAACCCTTCCCGTGACGTGGC  157
              |   |||||| |||||||||||||||||||||||||||| | ||||||||||||||
SelA  2315    -G---AAGACCAGTTCAGCAAGGTTATCTTCCAGCAGTGAAACGCGTCCCGTGACGTGGC  2260

PSong9  158   TGGCGGCGTGGTGAATATGCAGCGGCTGCCACTGGGTCAGCGGTGTATGGGTTTGAAGCT  217
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  2259    TGGCGGCGTGGTGAATATGCAGCGGCTGCCACTGGGTCAGCGGTGTATGGGTTTGAAGCT  2200

PSong9  218   CGCCAATCACCCGTGTGAACGGCTCTGGCGGCACATCGGCAAGCAGCCAGTCGCCACGGC  277
              || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SelA  2199    CGACAATCACCCGTGTGAACGGCTCTGGCGGCACATCGGCAAGCAGCCAGTCGCCACGGT  2140

PSong9  278   TAATCTGCTCTTTTTCCGCATCACCCGCGATGTTCAA-CGCGAAACGCTGCCCGGCATTG  336
              |||||||||||||||||||||||||||||||||| || ||||| ||||||||||||||||
SelA  2139    TAATCTGCTCTTTTTCCGCATCACCCGCGATGTT-AAGCGCGATACGCTGCCCGGCATTG  2081

PSong9  337   GCGGTTTCTGTTGGCTGGTTTTGCGCATGCACCGCACGTACACCCATCCGTTTATTTACA  396
              |||||||||||||||||||||||||||||||| |||||||||| |||| ||||||||||||
SelA  2080    GCGGTTTCTGTTGGCTGGTTTTGCGCATGCAGCGCACGTACACGCATCGGTTTATTTACA  2021

PSong9  397   CCAGTCAGCCATACTGAATCGCCTACCTTCACTTCCCCGCTTCACGACATACCGGTTAAA  456
              |||||||||| | |||||||||||||||||||||||||||| ||| | ||||||| |
SelA  2020    CCAGTCAGCCAGAGTGAATCGCCTACCTTCACTTCCCCGCTTAACGCCGTACCGGTGACG  1961

PSong9  457   ACTAAATCATCACATTTTACGGTAAATGCCCGGTTAATCACGAGGTGGA  505
              || |   |  ||| ||||||||||||||| |||| |||| ||||| |||
SelA    1960  ACCAGCCCGGCACCTTTTACGGTAAATGCGCGGTCAATCGCGAGGCGGA
```

Table 10.10. pSong10 Sequencing Results

Direction: Forward

```
pSong10  1    CATTAGGCAGCTCT-ATGCGCTGTT-ATCACTTTACTTTTATCTAATCTAGACATCATTA  58
              |||||  ||||||||  ||||||||||  ||||||||||||||||||||||||||||||||||
Tetprom  842  CATTAAGCAGCTCTAATGCGCTGTTAATCACTTTACTTTTATCTAATCTAGACATCATTA  901

pSong10  59   ATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGT  118
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetprom  902  ATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGT  961
                  SDS   HindIII
pSong10  119  GATAGAGAAAAAGCTT  135
              ||||||||||
Tetprom  962  GATAGAGAAA------  972


PSong10  136   ATGAGTATGTCAACATCCACTGAAGTCATCGCTCATCACTGGGCATTCGCTATCTTTCTT  195
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     958   ATGAGTATGTCAACATCCACTGAAGTCATCGCTCATCACTGGGCATTCGCTATCTTTCTT  899

PSong10  196   ATCGTTGCCATTGGCCTGTGTTGCCTGATGCTGGTAGGCGGTTGGTTTTTAGGCGGTCGC  255
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     898   ATCGTTGCCATTGGCCTGTGTTGCCTGATGCTGGTAGGCGGTTGGTTTTTAGGCGGTCGC  839

PSong10  256   GCACGCGCGAGGTCGAAAAACGTGCCGTTTGAATCCGGTATCGACTCGGTCGGCTCCGCC  315
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     838   GCACGCGCGAGGTCGAAAAACGTGCCGTTTGAATCCGGTATCGACTCGGTCGGCTCCGCC  779

PSong10  316   CGCTTACGCCTGTCCGCCAAGTTTTATCTGGTGGCCATGTTCTTCGTTATCTTCGACGTT  375
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     778   CGCTTACGCCTGTCCGCCAAGTTTTATCTGGTGGCCATGTTCTTCGTTATCTTCGACGTT  719

PSong10  376   GAAGCGCTGTATCTGTTCGCATGGTCAACCTCTATCCGCGAAAGCGGCTGGGTAGGCTTT  435
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     718   GAAGCGCTGTATCTGTTCGCATGGTCAACCTCTATCCGCGAAAGCGGCTGGGTAGGCTTT  659

PSong10  436   GTGGAAGCTGCAATTTTTATTTTTGTGTTACTGGCAGGTCTGGTTTATCTGGTGCGTATT  495
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     658   GTGGAAGCTGCAATTTTTATTTTTGTGTTACTGGCAGGTCTGGTTTATCTGGTGCGTATT  599

PSong10  496   GGCGCGCTGGACTGGACGCCCGCGCGTTCACGCCGCGAGCGTATGAACCCGGAAACGAAC  555
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     598   GGCGCGCTGGACTGGACGCCCGCGCGTTCACGCCGCGAGCGTATGAACCCGGAAACGAAC  539

PSong10  556   AGTATCGCTAATCGTCAACGCTAACCGCGAGGCATTAAGATGGATTATACGCTCACCCGC  615
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     538   AGTATCGCTAATCGTCAACGCTAACCGCGAGGCATTAAGATGGATTATACGCTCACCCGC  479

PSong10  616   ATAGATCCCAACGGTGAGAACGACCGTTACCCCCTGCAAAAGCAGGAGATCGTAACCGAC  675
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     478   ATAGATCCCAACGGTGAGAACGACCGTTACCCCCTGCAAAAGCAGGAGATCGTAACCGAC  419

PSong10  676   CCTCTGGAGCAAGAAGTTAACAAAAACGTGTTTATGGGCAAGCTCAATGACATGGTTAAC  735
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     418   CCTCTGGAGCAAGAAGTTAACAAAAACGTGTTTATGGGCAAGCTCAATGACATGGTTAAC  359

PSong10  736   TGGGGTCGTAAAAACTCAATTTGGCCGTATAACTTCGGTCTTTCCTGCTGTTACGTTGAG  795
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     358   TGGGGTCGTAAAAACTCAATTTGGCCGTATAACTTCGGTCTTTCCTGCTGTTACGTTGAG  299

PSong10  796   ATGGTGACTTCGTTTACCGCGGTGCATGACGTGGCGCGTTTTGGCGCAGAAAGTATTGCG  855
              |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
NuoA     298   ATGGTGACTTCGTTTACCGCGGTGCATGACGTGGCGCGTTTTGGCGCAGAA-GTATTGCG  240

PSong10  856   TGCTTCGCCGCGTCAGGCTGACCTGATGGTGGTTGCAGGAACCTGCTTTACCAAAATGGC  915
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     239   TGCTTCGCCGCGTCAGGCTGACCTGATGGTGGTTGCAGGAACCTGCTTTACCAAAATGGC  180

PSong10  916   ACCGGTTATTCAGCGTCTGTATGACCAGATGCTGGAACCAAAAATGGGTTTATCTCAATG  975
              |||||||||||||||||||||||||||||||||||||||||||| |||||| |||||||||
NuoA     179   ACCGGTTATTCAGCGTCTGTATGACCAGATGCTGGAACCAAAA-TGGGTT-ATCTCAATG  122
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PSong10  976   GGTGCCTGTGCCAACTCTGGGTGGTATGTACGATATTTATTCCGTTGTGCAGG-CGTCGA  1034
               |||||||||||||||||||| |||||||||||||||||||||||||||||| ||||||
NuoA     121   GGTGCCTGTGCCAACTCTGG-TGGTATGTACGATATTTATTCCGTTGTGCAGGGCGTCGA  63

PSong10  1035  TTAATTTCATCC-GGTT  1050
               | |||| ||||| ||||
NuoA     62    TAAATT-CATCCCGGTT  47
```

Direction: Backward

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PSong10  6     AGT-ATCGCTCATCACTGGGCATTCGCTATCTTTCTTATCGTTGCCATTGGCCTGTGTTG  64
               ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     935   AGTCATCGCTCATCACTGGGCATTCGCTATCTTTCTTATCGTTGCCATTGGCCTGTGTTG  876

PSong10  65    CCTGATGCTGGTAGGCGGTTGGTTTTTAGGCGGTCGCGCACGCGCGAGGTCGAAAAACGT  124
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     875   CCTGATGCTGGTAGGCGGTTGGTTTTTAGGCGGTCGCGCACGCGCGAGGTCGAAAAACGT  816

PSong10  125   GCCGTTTGAATCCGGTATCGACTCGGTCGGCTCCGCCCGCTTACGCCTGTCCGCCAAGTT  184
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     815   GCCGTTTGAATCCGGTATCGACTCGGTCGGCTCCGCCCGCTTACGCCTGTCCGCCAAGTT  756

PSong10  185   TTATCTGGTGGCCATGTTCTTCGTTATCTTCGACGTTGAAGCGCTGTATCTGTTCGCATG  244
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     755   TTATCTGGTGGCCATGTTCTTCGTTATCTTCGACGTTGAAGCGCTGTATCTGTTCGCATG  696

PSong10  245   GTCAACCTCTATCCGCGAAAGCGGCTGGGTAGGCTTTGTGGAAGCTGCAATTTTTATTTT  304
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     695   GTCAACCTCTATCCGCGAAAGCGGCTGGGTAGGCTTTGTGGAAGCTGCAATTTTTATTTT  636

PSong10  305   TGTGTTACTGGCAGGTCTGGTTTATCTGGTGCGTATTGGCGCGCTGGACTGGACGCCCGC  364
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     635   TGTGTTACTGGCAGGTCTGGTTTATCTGGTGCGTATTGGCGCGCTGGACTGGACGCCCGC  576

PSong10  365   GCGTTCACGCCGCGAGCGTATGAACCCGGAAACGAACAGTATCGCTAATCGTCAACGCTA  424
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     575   GCGTTCACGCCGCGAGCGTATGAACCCGGAAACGAACAGTATCGCTAATCGTCAACGCTA  516

PSong10  425   ACCGCGAGGCATTAAGATGGATTATACGCTCACCCGCATAGATCCCAACGGTGAGAACGA  484
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     515   ACCGCGAGGCATTAAGATGGATTATACGCTCACCCGCATAGATCCCAACGGTGAGAACGA  456

PSong10  485   CCGTTACCCCCTGCAAAAGCAGGAGATCGTAACCGACCCTCTGGAGCAAGAAGTTAACAA  544
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     455   CCGTTACCCCCTGCAAAAGCAGGAGATCGTAACCGACCCTCTGGAGCAAGAAGTTAACAA  396

PSong10  545   AAACGTGTTTATGGGCAAGCTCAATGACATGGTTAACTGGGGTCGTAAAAACTCAATTTG  604
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     395   AAACGTGTTTATGGGCAAGCTCAATGACATGGTTAACTGGGGTCGTAAAAACTCAATTTG  336

PSong10  605   GCCGTATAACTTCGGTCTTTCCTGCTGTTACGTTGAGATGGTGACTTCGTTTACCGCGGT  664
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     335   GCCGTATAACTTCGGTCTTTCCTGCTGTTACGTTGAGATGGTGACTTCGTTTACCGCGGT  276

PSong10  665   GCATGACGTGGCGCGTTTTGGCGCAGAAGTATTGCGTGCTTCGCCGCGTCAGGCTGACCT  724
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     275   GCATGACGTGGCGCGTTTTGGCGCAGAAGTATTGCGTGCTTCGCCGCGTCAGGCTGACCT  216

PSong10  725   GATGGTGGTTGCAGGAACCTGCTTTACCAAAATGGCACCGGTTATTCAGCGTCTGTATGA  784
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     215   GATGGTGGTTGCAGGAACCTGCTTTACCAAAATGGCACCGGTTATTCAGCGTCTGTATGA  156

PSong10  785   CCAGATGCTGGAACCAAAATGGGTTATCTCAATGGGTGCCTGTGCCAACTCTGGTGGTAT  844
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     155   CCAGATGCTGGAACCAAAATGGGTTATCTCAATGGGTGCCTGTGCCAACTCTGGTGGTAT  96
                                                                  SalI
PSong10  845   GTACGATATTTATTCCGTTGTGCAGGGCGTCGATAAATTCATCCCGGTTGAGTCGAC    900
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA     95    GTACGATATTTATTCCGTTGTGCAGGGCGTCGATAAATTCATCCCGGTTGA------    45

pSong10 901   CTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAAGTG  958
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
PBlue   58    CTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTG  1
```

Table 10.11. pLBRSong10 sequencing results

Direction: Forward

```
PLBRSong10  1    GACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGG  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PLBR322     89   GACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGG  30
                                                 SalI
PLBRSong10  61   GTTGAAGGCTCTCAAGGGCATCGGTCGAC GTCGAC 89
                 ||||||||||||||||||||||||||||||
PLBR322     29   GTTGAAGGCTCTCAAGGGCATCGGTCGAC ------ 1

PLBRSong10  90    AATGGGAATTGACGTTCCTTCCAAACATTCACGTAAAATCTGTTGTGCGTGTTTAGATTG  149
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro      82    AATGGGAATTGACGTTCCTTCCAAACATTCACGTAAAATCTGTTGTGCGTGTTTAGATTG  141

PLBRSong10  150   GAGTGAACGCCGTTTCCATTTAGGTGGGTACGTTGGAGCCGCATTATTTTCGCTTTATGA  209
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro      142   GAGTGAACGCCGTTTCCATTTAGGTGGGTACGTTGGAGCCGCATTATTTTCGCTTTATGA  201

PLBRSong10  210   ATCTAAAGGGTGGTTAACTCGACATCTTGGTTACCGTGAAGTTACCATCACGGAAAAAGG  269
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro      202   ATCTAAAGGGTGGTTAACTCGACATCTTGGTTACCGTGAAGTTACCATCACGGAAAAAGG  261

PLBRSong10  270   TTATGCTGCTTTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGCATATGATC  329
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro      262   TTATGCTGCTTTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGCATATGATC  321

PLBRSong10  330   AATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGCT  389
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro      322   AATTCAAGGCCGAATAAGAAGGCTGGCTCTGCACCTTGGTGATCAAATAATTCGATAGCT  381

PLBRSong10  390   TGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTCTTCTTTAGCGACT  449
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Tetpro      382   TGTCGTAATAATGGCGGCATACTATCAGTAGTAGGTGTTTCCCTTTCTTCTTTAGCGACT  441

PLBRSong10  450   TGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAA  488
                  ||||||||||||||||||||||||||||||||||||||||
Tetpro      442   TGATGCTCTTGATCTTCCAATACGCAACCTAAAGTAAAA  480
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Direction: Backward

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          BamHI
PLBRSO10 7GGATCC TCAACCGGGATGAATTTATTCTACTGCCCTTGCACAACGGAATAAATATCTGTACATACC  66
                 |||||||||||||||||| || || |||| |||||||||||||||||||| |||||||||
NuoA   914------ TCAACCGGGATGAATTTA-TCGAC-GCCC-TGCACAACGGAATAAATATC-GTACATACC  859

PLBRSong10  67   ACCAGAGTTGGCACAGGCACCCATTGAGATAACCCATTTTGGTACCAGCATCTGGTCATA  126
                 |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
NuoA  858        ACCAGAGTTGGCACAGGCACCCATTGAGATAACCCATTTTGGTTCCAGCATCTGGTCATA  799

PLBRSong10  127  CAGACGCTGAATAACCGGTGCCATTTTGGTAAAGCAGGTTCCTGCAACCACCATCAGGTC  186
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NuoA  798        CAGACGCTGAATAACCGGTGCCATTTTGGTAAAGCAGGTTCCTGCAACCACCATCAGGTC  739

PLBRSong10  187  AGCCTGACGCGGCGAAACACGCAATACTTCTGCGCCAAAACGCGCCACGTCATGCACCGC  246
                 ||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
NuoA  738        AGCCTGACGCGGCGAAGCACGCAATACTTCTGCGCCAAAACGCGCCACGTCATGCACCGC  679

PLBRSong10  247  GGTAAACGAAGTCACCATCTCAACGTAACATCTGGAAAGACCGAAGTTTTACGGCCCAAT  306
                 |||||||||||||||||||||||||||||| | |||||||||||||| ||||||| |||
NuoA  678        GGTAAACGAAGTCACCATCTCAACGTAACAGCAGGAAAGACCGAAGTTATACGGCCAAAT  619

PLBRSong10  307  TGAGTTTTTACAACCCCAGTTAACCATGTCATTGAGCTTGCCAATAAACACGTTTTTGTT  366
                 |||||||||||| |||||||||||||||||||||||||||||| ||||||||||||||||
NuoA  618        TGAGTTTTTACGACCCCAGTTAACCATGTCATTGAGCTTGCCCATAAACACGTTTTTGTT  559

PLBRSong10  367  AACTTCTTGCTCCAGAGGGTCGGTTACGATCTCCTGCTTTTGAAAGGGGTAACGGACGTT  426
                 |||||||||||||||||||||||||||||||||||||||||||| | |||||||||| ||||
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NuoA  558         AACTTCTTGCTCCAGAGGGTCGGTTACGATCTCCTGCTTTTGCAGGGGGTAACGGTCGTT  499

PLBRSong10  427   CTCACCGTTGGGATCTATGCAGGTGAGAGTATAATCCATCTTAATGCCTCGCGGTTACCG  486
                  |||||||||||||||||||| |||||| |||||||||||||||||||||||||||| ||
NuoA  498         CTCACCGTTGGGATCTATGCGGGTGAGCGTATAATCCATCTTAATGCCTCGCGGTTAGCG  439

PLBRSong10  487   TTGACCATTAGCCATACTGCTCGTTTCCGGTTTTCATACGCTCGCGGCGTGATTGCGCCG  546
                  ||||| |||||| |||||| |||||||||| || ||||||||||||||||||  |||| |
NuoA  438         TTGACGATTAGCGATACTGTTCGTTTCCGGGTT-CATACGCTCGCGGCGTGAACGCGCGG  380

PLBRSong10  547   -CGTCCAGTCCAGCGCGCCAA  566
                   ||||||||||||||||||||
NuoA        379    GCGTCCAGTCCAGCGCGCCAA  359
```